NITRIC OXIDE-MEDIATED SIGNALING IN PULMONARY ENDOTHELIAL CELLS

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S-nitrosothiol modifications of proteins are emerging as an important nitric oxide-mediated signaling pathway. Our laboratory has focused on S-nitrosation of the metal binding protein metallothionein and the resulting effects on zinc homeostasis, gene and protein expression and nitric oxide (NO) mediated signaling in the pulmonary endothelium. *Statement of public health significance:* The pulmonary endothelium is responsible for filtering the blood before it enters systemic circulation, and as such it is extremely vulnerable to injury by inhaled toxicants in the environment as well as those that circulate in the bloodstream. As the endothelium constitutively produces NO, we are interested in studying NO-mediated signaling in order to lay a foundation that will allow us to better understand diseases such as asthma, pulmonary hypertension and sepsis in which dysregulation of NO-mediated signaling is thought to be a contributing factor to the disease pathology.

To this end we have used both recombinant DNA and biochemical techniques to examine the relationship between metallothionein, zinc homeostasis and the metal responsive transcription factor MTF-1. We demonstrated that exposure to NO results in zinc release from metallothionein, which in turn activates MTF-1, resulting in nuclear translocation of the protein and NO-dependent increases in metallothionein protein expression. We hypothesized that S-

nitrosation of the sulphydryl groups in metallothionein were the cause of NO-mediated zinc release and downstream protein expression effects. We used a fluorescent modification of the biotin switch assay in combination with two-dimensional electrophoresis and mass spectroscopy to extend our study of NO-mediated signaling through S-nitrosation of protein thiols to identify S-nitrosated metallothionein in endothelial cells exposed to NO donor, and used the technique in further studies to illuminate the proteome of pulmonary endothelial cells. We were able to identify several potential targets of S-nitrosation in endothelial cells including cytoskeletal, cytoprotective, glycolytic and chaperone proteins. The proteomic assay that we developed is a useful screening tool, and may lead to new insights in post-translational S-nitrosothiol modifications of endothelial proteins, and eventually to new perspectives regarding diseases exacerbated by dysregulation of this NO-mediated signaling pathway.

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PREFACE

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1.0 CHAPTER 1: INTRODUCTION

Nitric oxide is a gaseous, free radical molecule produced by nitric oxide synthase (NOS) that regulates a large number of biological functions including vasodilation, neurotransmission, inflammation and cell death [1]. Our goal in the following studies has been to examine the potential role of NO as a signaling molecule in the pulmonary endothelium. In our studies we have used a combination of molecular cloning, imaging and biochemical techniques to examine the potential connections between the low-molecular weight, thiol-rich protein metallothionein (MT), NO, and zinc homeostasis in the endothelium. We expanded upon these studies to screen cultured primary endothelial cells for proteins that may be involved in an emerging nitric oxide-mediated signaling pathway involving S-nitrosation of protein thiols. In order to complete this screening, we made modifications to an accepted S-nitrosothiol detection technique.

1.1 NITRIC OXIDE-MEDIATED SIGNALING IN THE PULMONARY ENDOTHELIUM AND PUBLIC HEALTH

Endothelial cells comprise the lining of all blood vessels. In the human lung, which has the largest combined endothelial/epithelial surface areas of any organ, the endothelium has a surface area of approximately 130 m^2 [2; 3]. The primary functions of the endothelium are to promote anti-aggregation, serve as a barrier, and to synthesize, metabolize or take up vasoactive

compounds [4]. As such, the pulmonary endothelium comes into contact with the entire contents of circulating blood as well as being exposed to any inhaled particles or toxicants. Toxicological insults to the endothelial layer as well as clinical disorders such as sepsis, and pneumonia can lead to acute lung injury [5]. Symptoms of acute lung injury include severe respiratory distress and hypoxia [4]. In certain cases acute lung injury may progress to a more severe form of disease, acute respiratory distress syndrome, which is characterized by development of pulmonary edema, and increases in both reactive oxygen and reactive nitrogen species. It is estimated that acute respiratory distress syndrome may account for up to 36 000 deaths per year in industrialized nations [6]. It is known that production of reactive oxygen and nitrogen species is increased in acute respiratory distress syndrome, and that one source of reactive nitrogen species is the pulmonary endothelium itself [7]. There is histochemical evidence of reactive nitrogen species-based protein modifications in samples from the lungs of acute respiratory distress syndrome patients [7; 8]. It has also been shown that NO can attenuate leakage in the lungs of patients with acute respiratory distress syndrome, and that therapeutic inhaled NO can abate hypoxia in both animal models and human studies [9].

It is known that sepsis is the most significant risk factor for development of acute lung injury or acute respiratory distress syndrome [10; 11]. The mortality rate for sepsis ranges from 15% to >60% in septic shock [12]. It has been demonstrated that NO production in endothelial cells is increased in sepsis, perhaps through expression of iNOS [13; 14; 15; 16; 17]. Increased NO production may explain the drop in blood pressure observed in sepsis [18] that can be reversed with NOS inhibitors [19; 20; 21]. In addition increased NO production may lead to the formation of peroxynitrite (ONOO-). Decomposition of ONOO- into peroxide radicals ($HO^{\bullet}$) may result in endothelial cell death [22]. However, it has also been demonstrated that exposure

to low levels of NO can inhibit LPS-induced apoptosis [23; 24; 25; 26]. It has been speculated that suppression of LPS-induced apoptosis occurs through inactivation of caspase-3 [23; 26], which has been demonstrated to be S-nitrosated at a critical catalytic residue [27; 28; 29]. However the endothelial signaling pathways that are involved in acute lung injury and acute respiratory distress syndrome are incompletely understood and the development of new screening tools to examine potential dysregulation of S-nitrosothiol-based signaling would be extremely valuable in dissecting the mechanisms that lead to onset and progression of injury.

In addition to potential involvement in sepsis and acute lung injury, it has been suggested that dysregulation of NO-mediated signaling pathways is involved in many other diseases [30]. Asthma is one such disease. In an ovalbumin-sensitization animal model of asthma, metabolism of S-nitrosoglutathione (GSNO) is increased leading to low levels of GSNO in the airway lining fluid [31]. Indeed, levels of GSNO in the airway lining fluid of human asthma patients are low, even though conditions would be expected to favor S-nitrosothiol formation [32; 33]. In addition, in pulmonary hypertension investigators found levels of S-nitrosohemoglobin to be reduced relative to normal controls [30]. From these selected examples, it is clear that dysregulation of S-nitrosothiol homeostasis contributes to disease states and that a tool for examining the spectrum of S-nitrosation in pulmonary endothelial cells would be very useful.

1.2 CELLULAR PRODUCTION OF NITRIC OXIDE

NO plays a variety of roles in biological systems and is produced in various cell types by an enzyme, nitric oxide synthase (NOS). All NOS isoforms catalyze the five-electron oxidation of the terminal nitrogen of arginine, using NADPH, flavins, tetrahydrobiopterin, and thiol as co-

factors, ultimately producing nitric oxide (NO) and citrulline [34]. It is believed that successive activation of two O_2 molecules by the heme moiety of NOS is needed to insert a pair of oxygen atoms into arginine and yield NO [34]. It is also known that under certain cellular redox conditions NOSs can produce NO^- [34].

The first isoform, eNOS, is constitutively expressed primarily in endothelial cells in the vasculature, and the nitric oxide synthesized results in vasorelaxation [35]. The second isoform, nNOS, is constitutively expressed in the nervous system where it functions as a neurotransmitter [36]. Finally, iNOS expression is induced in macrophages as a protective mechanism in inflammation in the immune system [36]. Since our studies have focused on signaling in the endothelium, it is necessary to touch upon the regulation of NO production in eNOS.

There are three known levels of regulation for eNOS; 1) the interaction of eNOS with proteins such as caveolin and Ca^{2+}/calmodulin, 2) the phosphorylation state of the enzyme and, 3) the differential location of the enzyme within the cell [37]. eNOS seems to be regulated differently depending upon its subcellular location. At the plasma membrane, Ca2+/calmodulin regulates eNOS, whereas in the Golgi regulation occurs mainly through Akt [38]. Within the cell, trafficking of eNOS appears to be dependent upon the actin cytoskeleton in addition to interactions with proteins such as eNOS-trafficking inducer (NOSTRIN) and caveolin [37].

It is also known that interactions of eNOS with the cellular cytoskeleton, particularly actin microfilaments, are essential for shear stress-mediated NO production [39]. eNOS protein interacts directly with the actin cytoskeleton, and disruption of this interaction via changes in the cytoskeletal structure during shear stress results in an increase in eNOS mRNA half-life and ultimately in increased NO production [39].

1.3 BIOCHEMISTRY OF NITRIC OXIDE

Upon synthesis by NOS, NO chemistry involves a number of redox forms: nitrosonium cation (NO^+), nitric oxide ($NO^\bullet$), which is a free radical species, and the nitroxyl anion (NO^-) [34]. NO is a paramagnetic molecule with a single, unpaired electron in its 2p-π orbital (Figure 1.1) [40].

$$N::O^\bullet$$

Figure 1-1 Nitric oxide is a paramagnetic molecule. Its unique biochemical properties can be attributed to the presence of a single, unpaired electron in the 2p-π orbital.

Addition of an electron to NO leads to formation of NO^- while loss of an electron results in formation of NO^+ [34]. NO reacts quite readily with oxygen (O_2) and superoxide ($O_2^\bullet$) to form various nitrosocompounds and peroxynitrite ($ONOO^-$) [34]. NO can also react with transition metals including heme moieties. Reaction of NO with heme results in formation of an iron-nitrosyl complex. Upon binding the $Fe^{(III)}NO^{\bullet-}$ heme undergoes a charge transfer to form $Fe^{(II)}NO^+$ making the NO^+ moiety available for attack by cellular nucelophiles [41]. This property allows NO-transition metal complexes to function as effective "carriers" of NO^+ in the cell [34]. Upon formation of an NO^+ equivalent various cellular nucleophiles such as amines and thiols may attack the NO^+ and become nitrosated. Under physiological conditions S-nitrosation (the addition of a NO^+ moiety to a thiol) is favored over N-nitrosation (the addition of a NO^+ moiety to an amine) due to the existence of amines primarily in their unreactive, protonated form [42]. In addition S-nitrosation is favorable due to the lower rate of hydrolysis of S-nitrosothiols as compared to nitrosamines [42]. The second redox species of NO, NO^-, is known to be

converted to N_2O in aqueous solution, react with $Fe^{(III)}$ heme, and to oxidize cellular thiols, ultimately resulting in the formation of disulfides [34].

1.4 METALLOTHIONEIN, NITRIC OXIDE AND ZINC HOMEOSTASIS

Metallothioneins (MT) are small (6 kDa), cysteine-rich, heavy metal binding proteins [43] (Figure 1.2). There are four known MT isoforms in mice, each with a particular expression pattern. MT-I and MT-II are expressed in the central nervous system and stratified squamous epithelium, respectively [44] and these isoforms account for most of the effects of MT. Several functions have been suggested for MT including detoxification of heavy metals, regulation of copper and zinc homeostasis and protection from oxidative damage [45; 46]. There is in vitro data to support the suggestion that MT forms a link between cellular redox state and metal ion homeostasis [47; 48; 49; 50; 51; 52; 53].

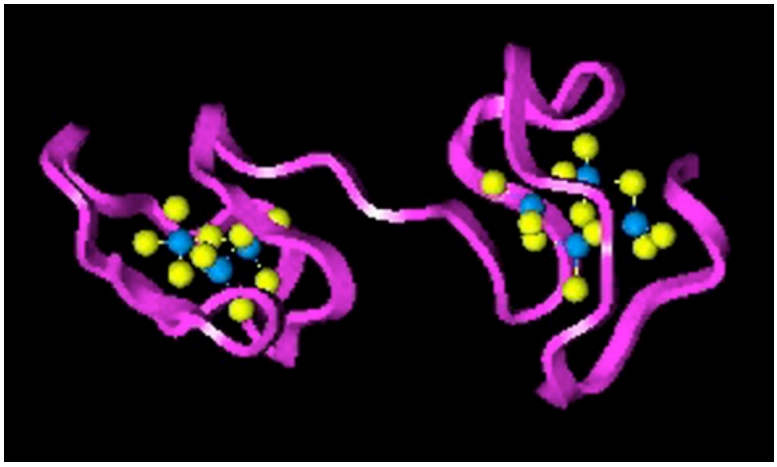

Figure 1-2 Metallothionein. Metallothionein contains 61 amino acid residues, 20 of which are cysteines. It can bind 7 divalent metal atoms, 3 in the N terminal or alpha cluster and 4 in the C terminal or beta cluster. In this image cysteine residues are represented in yellow and metal ions in blue [54].

There is evidence that MT can react with NO forming iron-dinitrosyl-sulfur complexes that can be detected using electron paramagnetic resonance (EPR) [54; 55]. In addition there is evidence that exposure to NO can result in the release of metals such as cadmium [56], copper [57; 58], and zinc [59; 60; 61; 62]. Our laboratory has focused on interactions between NO and MT in the vascular endothelium. We have used various techniques to study this interaction including the use of an MT fusion protein consisting of MT sandwiched between cyan and yellow fluorescent protein and fluorescence resonance energy transfer (FRET) and various Zn-specific fluorophores [60; 61; 62]. In these studies we demonstrated that exposure to NO caused unfolding of MT [60; 61] and increases in intracellular Zn^{2+} concentration [62], presumably through S-nitrosation of thiol residues in MT. Indeed other groups have found that the thiol moieties in MT can be S-nitrosated in vitro [63]. In this study we have extended our previous work describing the effect of MT and NO-mediated zinc homeostasis in vascular endothelial cells to the level of transcriptional regulation through the metal response element binding transcription factor, MTF-1.

1.5 METAL RESPONSE ELEMENT BINDING TRANSCRIPTION FACTOR MTF-1, NITRIC OXIDE AND METALLOTHIONEIN

MTF-1 is a highly conserved, constitutively expressed, Zn-finger protein in the Cys_2-His_2 family of transcription factors [64; 65; 66; 67]. MTF-1 is an essential molecule as knockout mice suffer from embryonic lethality [68]. MT-I and MT-II gene expression is dependent upon the presence of MTF-1 in the cell [67]. The Cys_2-Hys_2 zinc finger motifs are involved in both tetrahedral

coordination of a Zn^{2+} atom (Figure 1.3A), while other residues are involved in binding to the major groove of DNA (Figure 1.3B) [67]. MTF-1 binds to metal responsive elements (MREs) in promoters of zinc responsive genes [67].

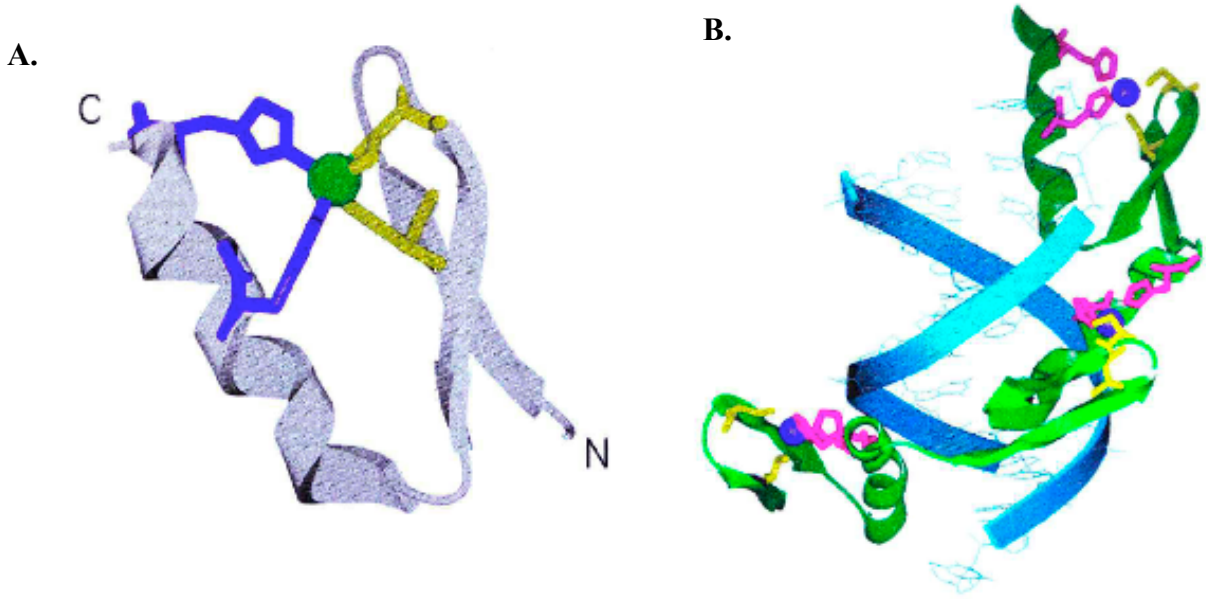

Figure 1-3 A representative Cys_2-His_2 transcription factor. Structure of Cys_2-His_2 zinc fingers in a representative transcription factor Zif268. A) Representation of the Cys and His residues that provide coordination of a Zn^{2+} atom B) Residues that are involved in binding to the major groove of DNA.

Some of the best studied MREs are present in the MT-I and MT-II promoters. MREs are often arranged in tandem in the promoters of zinc-regulated genes and contain a number of 5'-CpG sequences [67]. Heavy metals such as zinc and cadmium stimulate nuclear translocation of MTF-1 in mouse and human cells. Once in the nucleus, MTF-1 binds to MREs and activates transcription of target genes such as MT [66], as well as other target genes such as zinc transporter 1 (ZnT1) [69], gamma-glutamylcysteine synthase heavy chain [70], and placental growth factor [71].

It has been suggested that release of Zn by reactive oxygen or nitrogen species could cause activation of genes transcribed by MTF-1 and act as a novel signal transduction pathway to protect cells against oxidative stress [72; 73; 74]. Our laboratory has demonstrated that MT plays a critical role in the regulation of zinc homeostasis via interactions with NO [25; 62] (See Appendix A). In addition, iNOS derived NO and NO donors have been shown to increase MT mRNA gene expression via intracellular zinc release [75].

In our current research we continued to analyze the relationship between NO-mediated zinc release from MT and MTF-1 activation. We created an EGFP-MTF-1 chimeric protein to allow us to use imaging techniques to study MTF-1 translocation in response to exposure to NO donors. We also used Western Blotting to examine changes in MTF-1 and MT protein after treatment with NO donors (See Chapter 2).

1.6 THE BIOCHEMISTRY OF S-NITROSATION

In our research we have been focused upon a particular subsection of NO biochemistry: its reaction with thiol groups known as S-nitrosation. Nitrosation is defined as the covalent attachment of an NO group to a transition metal or a thiol [76]. The attachment of an NO group to a cysteine thiol is known as S-nitrosation. S-nitrosated proteins can be detected in vivo and in the presence of NOS inhibitors [77; 78; 79; 80]. In most cases where the site of S-nitrosothiol modification has been identified for a particular protein it has been found to be a modification of a regulatory or active site thiol [81]. The exact biochemical mechanism of S-nitrosothiol formation in the cellular environment is still a subject of debate, however there are several widely accepted theories, which we will discuss here.

It is known that NO^+ itself does not exist in biological systems per se. Instead there are several species that can be thought of as "carriers" of NO^+ in the cell. These can be NO itself, nitrite, N_2O_3, metal-nitrosyl species or another cellular S-nitrosothiol [30]. The formation of an S-nitrosothiol involves the transfer of a NO^+ equivalent from a "carrier" species to a cysteine thiol [76]. To date there has been no enzyme that has been demonstrated to be involved in the creation of S-nitrosothiols [81]. There have been several proteins that have been implicated in promotion of S-nitrosation and denitrosation reactions [82; 83; 84; 85; 86; 87; 88; 89; 90]. One example of a protein that can decompose S-nitrosothiols is glutathione-dependent formaldehyde reductase [84]. Researchers found that this enzyme, which is conserved from bacteria to humans, can regulate intracellular levels of both S-nitrosoglutathione (GSNO) and other protein S-nitrosothiols [84]. Another protein that has been demonstrated to have S-nitrosothiol reducing capability is protein disulfide isomerase (PDI) [87]. PDI is excreted from cells that produce NO, such as endothelial cells, and the reduced form of PDI is capable of denitrosating both GSNO and S-nitrosated PDI [87].

1.7 S-NITROSATION AS A NEWLY EMERGING SIGNALING PATHWAY

S-nitrosation of protein thiols has been described as a newly emerging redox-based cellular signaling pathway [81]. It has been compared to protein phosphorylation albeit with several important differences. S-nitrosation signaling is a cGMP-independent process and there is, as of yet, no specific nitrosating enzyme has been identified leading to the conclusion that S-nitrosation a redox chemistry-mediated signaling pathway [76]. In general it is thought that the formation of protein S-nitrosothiols will depend upon the relative formation and colocalization of

reduced thiols, thiyl radicals, NO and NO^+ equivalents in the cell [76]. There are several factors that can lend specificity to this redox reaction-based signaling pathway.

The first possible factor that can increase the specificity of S-nitrosation reactions is the pKa of the thiol target [81]. It has been hypothesized that flanking acidic (Asp, Glu) and basic (Arg, His, Lys) residues in a protein's primary amino acid sequence, tertiary or quaternary structure may contribute to changes in the target thiol's pKa, rendering it more susceptible to S-nitrosation [91]. There is controversy in the literature as to the relevance of the theory of an "acid-base motif". Several groups claim to have identified modified cysteine thiols using this motif in combination with mass spectroscopy-based methods [92; 93]. However, another group using a computer-learning based approach based upon the results of mass spectroscopic analysis failed to find a relationship between linear amino acid sequence and S-nitrosation of a specific protein thiol residue [94]. Therefore, it seems that if the "acid-base motif" exists, it is most likely based upon the presence of pKa altering residues in a protein's tertiary or quaternary structure.

Another possible mechanism leading to increased specificity of S-nitrosation involves the presence of hydrophobic pockets surrounding susceptible thiol residues within a protein [91]. It is known that reactions between NO and O_2 are accelerated in hydrophobic pockets and can lead to increased production of NO_2 and N_2O_3, both of which can function as NO^+ equivalents [95; 96]. There are several examples of proteins in which this mechanism is thought to be important in driving specificity of S-nitrosation [91; 96; 97; 98]. In a particular example, redox-driven conformational changes in the ryanodine receptor generate a hydrophobic pocket surrounding the critical cysteine residue that is modified by S-nitrosation [91; 97].

A third factor that may influence specificity of S-nitrosation is colocalization of the target protein with NOS itself. In cases where a protein may directly interact with a NOS isoform it has been determined that specificity of S-nitrosothiol modifications may be increased. One of the best-characterized examples of this type of interaction is that of the N-methyl-D-aspartate receptor (NMDAR) in neurons. In neurons, nNOS is held at the membrane by interactions with various scaffold-binding proteins [99]. The NMDAR also interacts with some of these same proteins, bringing it into close proximity with nNOS, making the NMDAR both a source of nNOS activation via initiation of Ca^{2+} flux, and a substrate for nNOS-dependent S-nitrosation [77; 100].

Zn^{2+} coordinated thiols more likely targets for S-nitrosation [101; 102]. Metallothionein (MT), with its 20 cysteine residues capable of coordinating up to 7 divalent metal ions (including Zn^{2+}) [103] makes (MT) a tempting target for S-nitrosation. Previous studies in our laboratory have examined the interaction between NO and zinc release from the thiol groups in MT [60; 61; 62]. In these previous studies we have hypothesized that S-nitrosation of the thiol residues in MT via exposure to NO donors is responsible for the conformational changes and zinc release that we observed. In this study we have used a modification of a S-nitrosothiol detection technique to examine the S-nitrosation status of MT in sheep pulmonary artery endothelial cells (SPAEC) (See Chapter 3).

1.8 MEASUREMENT OF S-NITROSOTHIOLS

There are several methods available to measure S-nitrosothiols, each with its own set of limitations that we will discuss. It is challenging to quantify S-nitrosothiols in biological

systems due to their very low in vivo levels (predicted to be in the nM range in most biological situations) [104], and interference from nitrite, iron-nitrosyl, and other nitroso compounds in S-nitrosothiol detection assays [105]. In addition, S-nitrosothiols can be decomposed by thiols, ascorbic acid and metals, which, if present, can cause loss of signal during sample processing [104]. The difficulties in accurate measurements of S-nitrosothiol levels can be illustrated by the wide range in values (from mM amounts down to nM amounts) reported by various laboratories using different methods of analysis [104; 106]. Not only is it important to use the proper measurement technique, but also proper sample preparation is key to preserve the S-nitrosothiols that are to be measured. We will now discuss some of the most common measurement techniques along with their potential pitfalls.

The first commonly used method to measure S-nitrosothiols involves the use of photolysis-chemiluminescence. Chemiluminescent detectors use the ability of NO to react with ozone to generate NO_2^* which decays back to NO_2 by releasing near-infrared radiation that can be detected by a photomultiplier [104]. This reaction occurs in the gas phase and it is important that NO and ozone are mixed in equal proportion in order to maximize the luminescence produced [107]. There are two commonly used methods to release NO from S-nitrosothiols, either photolysis or chemical reduction.

In photolysis-chemiluminescence, high-intensity UV light in the 300-350 nm range is used to activate S-nitrosothiols [107]. Following activation, the S-nitrosothiol can decompose to free NO and a thiyl radical. It is important to note that in photolysis experiments NO can be formed from nitrite present either in the sample to be analyzed or the buffers used if the pH is allowed to become too low, if UV light below 300 nm is used in the assay, or if thiols are present [107; 108]. In addition, photolysis can release NO from nitrosamine or dinitrosyl-iron

complexes in the sample [109]. If used properly the sensitivity of a photolysis system should be in the low nM-range.

In chemical reduction assays S-nitrosothiols can be cleaved using the triiodide method or by using Cu^{+} and cysteine [110; 111]. In the triiodide method samples are pre-treated with acidified sulfanilamide to eliminate reactivity of nitrite in the assay [112]. A mixture of I_2/I^- in acidic conditions cleaves the S-NO bond and S-nitrosothiols are measured as the difference of the chemiluminescent signal of samples pre-treated with $HgCl_2$ and untreated samples [105]. In the Cu^{+} and cysteine method the cysteine transforms all S-nitrosothiols into Cys-S-NO [110]. The resulting NO^{+} equivalent is reduced by Cu^{+} to form NO, and remaining cysteine reduces the Cu^{2+} formed in the reaction back to Cu^{+} [113].

Colorimetric assays for S-nitrosothiols are generally thought to be a relatively insensitive method for measurement due to the fact that the limit of detection of this assay is around 500 nM, which is relatively close to the biological concentration of S-nitrosothiols [114]. $HgCl_2$ is used to displace NO^{+} from the S-nitrosothiol, which is detected as nitrite by using Griess reagents [115].

There are fluorescence-based assays that use the transfer of NO^{+} equivalents to 4,5-diaminofluoresceine (DAF) resulting in formation of the fluorescent compound (DAF-2T) [107]. It is possible to use photolysis, CuCl, or $CuSO_4$ to facilitate transfer of NO^{+} equivalents between the S-nitrosothiol and DAF. There are some arguments that the presence of free thiols can cause NO and S-nitrosothiol formation from nitrite and can cause increased levels of artifactual fluorescence, rendering the assay inaccurate [108].

Mass spectrometry (MS)-based analysis of S-nitrosothiols is becoming a commonly used S-nitrosothiol measurement method due to its sensitivity and accuracy [104]. MS-based

approaches can be used in combination with liquid chromatography to directly detect the addition of a NO moiety to a thiol as long as the sample preparation is performed carefully [107]. Several laboratories have used MS-based approaches to identify substituted or "switched" S-nitrosothiols as well [77; 92; 94].

Finally, there is a method that has been used to detect individual S-nitrosoproteins in complex mixtures such as the cellular proteome. This method was developed by Jaffrey and his colleagues [77; 116] and is known as the Biotin Switch Assay.

1.9 A MODIFIED BIOTIN SWITCH METHOD FOR DETECTION OF PROTEIN S-NITROSOTHIOLS

In our studies we have used a modification of the original biotin switch method for S-nitrosothiol detection (Figure 1.4). In the original version of the Biotin Switch method protein samples can contain both reactive free thiols as well as S-nitrosothiols. The first step in the method involves blocking free-reactive thiols with an alkylating agent, methyl methane thiosulfonate (MMTS) in the presence of SDS to expose all thiols to treatment. In the assay, if free reactive thiols are incompletely blocked, there can be an increase in background levels of S-nitrosothiol detection [117].

One way to reduce background labeling from unblocked, free reactive thiols is to increase the number of blocking steps; in our case we use two blocking steps with MMTS. The next step in the original protocol involves reduction of protein S-nitrosothiols using ascorbate. However, it has been determined that S-nitrosothiols in proteins of various molecular weights have different rates of reaction with ascorbate [118].

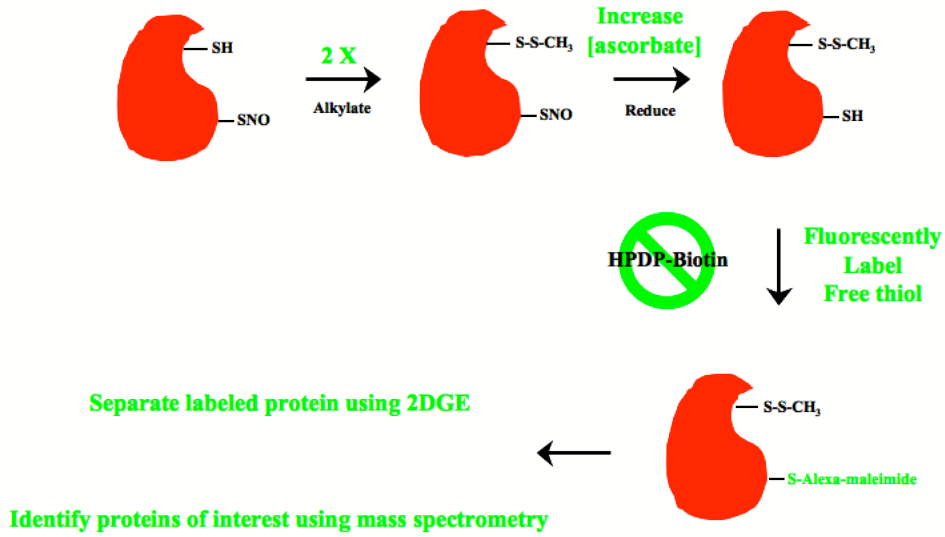

Figure 1-4 The Biotin Switch Method for detection of protein S-nitrosothiols. The steps from the original assay are noted in black. Modifications to the original assay are noted in green.

It is necessary to take this into consideration in order to effectively reduce all possible S-nitrosothiols. Failure to reduce S-nitrosothiols leads to decreased sensitivity in the assay. Increasing the ascorbate concentration and incubation time is one way to ensure efficient reduction of S-nitrosothiols (Figure 1.4) [118]. The next step of the original assay involves reacting the free, reactive thiol formed from the S-nitrosothiol via reduction with ascorbate with a thiol specific biotin moiety, *N*-[6(Biotinamido)-hexyl]-3'(2'-pyridyldithio)propionamide (Biotin-HPDP). In the original assay, S-nitrosated proteins are detected on a Western Blot using a secondary detection method such as an anti-biotin antibody or a labeled-streptavidin moiety. However, using secondary detection techniques complicates the use of proteomic techniques. Therefore in our research we replaced the Biotin-HPDP moiety with a thiol-reactant fluorophore (Alexa labeled maleimide, Invitrogen) in order to label S-nitrosoproteins so that they may be directly detected in two dimensional gel electrophoresis techniques (2DGE). We can then

identify S-nitrosated proteins of interest using mass spectroscopy and peptide mass fingerprinting.

There are several caveats that must be considered when using the Biotin Switch Method or a modification of the method. The first issue is that the Biotin Switch Method depends upon being able to identify a very small difference in the levels of S-nitrosothiols in the presence of a very large background of cellular thiols [117]. As an example of the potential for difficulty in detecting S-nitrosothiols the following scenario has been considered [117]: If the total cellular thiol level is approximately 100 nmol/mg protein, then in order to be able to detect 100 pmol/mg S-nitrosothiol with a signal-to-noise ratio of 10:1, then 99.99% of free protein thiols need to be blocked.

2.0 CHAPTER 2: NITRIC OXIDE-INDUCED NUCLEAR TRANSLOCATION OF THE METAL RESPONSIVE TRANSCRIPTION FACTOR, MTF-1, IS MEDIATED BY ZINC RELEASE FROM METALLOTHIONEIN

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Molly S. Stitt, K. J. Wasserloos, Z. Tang, X. Liu, Bruce R. Pitt and Claudette M. St. Croix. Nitric oxide-induced nuclear translocation of the metal responsive transcription factor, MTF-1, is mediated by zinc release from metallothionein. Vascular Pharmacology, 2006; 44: 149-155.

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2.2 INTRODUCTION

Previous studies in our laboratory have established a link between NO exposure and zinc release from MT [25; 60; 61; 62; 119; 120]. We have used a fluorescent chimeric MT molecule as an intracellular zinc reporter [60; 61]. In this case we hypothesized that exposure to NO caused S-nitrosation of the thiol residues in MT resulting in zinc release and conformational changes in the protein. We have also used zinc specific fluorophores to examine changes in intracellular zinc levels [25; 61; 62; 120]. We extended these studies by examining the transcriptional control of zinc homeostasis through MT and the metal responsive transcription factor MTF-1. To do this we created an EGFP-MTF-1 fusion protein and tracked nuclear translocation after exposure to NO donors. We also examined MTF-1 nuclear and cytoplasmic protein expression with and without exposure to NO donors via Western Blotting. Finally, we monitored NO donor-dependent MT protein expression levels.

2.3 MANUSCRIPT AS PUBLISHED IN VASCULAR PHARMACOLOGY

2.3.1 Abstract

We previously showed that the major Zn-binding protein, metallothionein (MT) is a critical target for nitric oxide (NO) with resultant increases in labile Zn. We now show that NO donors also affected the activity of the metal responsive transcription factor MTF-1 that translocates from the cytosol to the nucleus in response to physiologically relevant increases in intracellular Zn and transactivates MT gene expression. Exposing mouse lung endothelial cells (MLEC) to $ZnCl_2$ or the NO donor, S-Nitroso-N-acetylpenicillamine (SNAP, 200 μM), caused nuclear translocation of a reporter molecule consisting of enhanced green fluorescent protein (EGFP) fused to MTF-1 (pEGFP-MTF-1). In separate experiments, NO donors induced increases in MT protein levels (Western blot). In contrast, NO did not cause nuclear translocation of EGFP-MTF-1 in MLEC from MT knockouts, demonstrating a central role for MT in mediating this response. These data suggest that S-nitrosation of Zn-thiolate clusters in MT and subsequent alterations in Zn homeostasis are participants in intracellular NO signaling pathways affecting gene expression.

2.3.2 Introduction

Metallothionein (MT), with 20 cysteine residues, is the major zinc binding protein in most cells [121], and is a demonstrated molecular target for nitric oxide [59; 60; 62; 122] with associated changes in the intracellular concentration of labile zinc [59; 62; 75; 123]. While the precise nature of the NO-induced modification of MT is not clear, NO-based intermediates are

capable of inducing S-nitrosation of the sulphydryl groups [59; 124] resulting in loss of zinc from the metal binding centers of the protein [59]. The cysteines of metal thiolate clusters in MT can thereby confer unique redox sensitivity to an otherwise redox inert metal ligand (e.g. zinc) and facilitate the potential for MT to participate in intracellular signal transduction pathways *via* the regulation of intracellular zinc trafficking and homeostasis [53; 62; 75]. Zinc atoms are positioned at catalytic, structural and regulatory sites of enzymes (e.g. caspase-3, superoxide dismutase, NO synthase), transcription factors (TFIIIA) and structural proteins accounting for its importance in maintaining a wide variety of biological processes [51; 125], and providing a number of putative targets for MT-released zinc.

MT participates in a variety of protective stress responses in addition to its role in regulating zinc levels and distribution within the cell. In this regard, the Zn-sensitive, metal response element binding transcription factor, MTF-1 plays a central role in transcriptional activation of the MT gene in response to heavy metals and oxidative stress [74; 126]. MTF-1 is a highly conserved Zn-finger protein in the Cys_2His_2 family of transcription factors [64; 65; 66] that binds and transactivates gene expression through metal responsive elements (MREs) present in multiple copies in the proximal promoter of the MT I and MT II genes [66] as well as other target genes, including those for the zinc transporter, ZnT1 [69], gamma-glutamylcysteine synthase heavy chain [70] and placental growth factor [71]. It has been theorized that release of Zn by reactive oxygen/nitrogen species and activation of genes transcribed by MTF-1 could act as a novel signal transduction pathway, which protects the cell from oxidative stress [72; 73; 74].

Our original finding that MT was critical to the regulation of zinc homeostasis by NO [62; 120] was recently confirmed using cytokine induced increases in iNOS derived NO [75]. Furthermore, both iNOS-derived NO, and NO donors, were shown to increase MT mRNA gene

expression via intracellular zinc release [75]. In the present report, we extended these findings to show that NO-induced changes in labile zinc impact upon the activity of MTF-1 and increase expression of MT protein. Collectively, these data support a signaling pathway involving NO-mediated release of Zn from MT, zinc-sensitive activation of MTF-1, and altered expression of protective genes including MT.

2.3.3 Methods

Materials: Plasmid pCMV-MTF1 was provided courtesy of Glen Andrews. Cell culture reagents including DMEM, OptiMEM, and L-glutamine were purchased from Invitrogen. ENDO GRO was purchased from VEC Technologies. SNAP was purchased from Molecular Probes (Eugene, OR). The monoclonal mouse anti- metallothionein (MT) antibody was from Dako (Carpinteria, USA). The hydrochloride of S-nitroso-L-cysteine ethyl ester (SNCEE·HCl) was prepared via direct S-nitrosation of the hydrochloride of L-cysteine ethyl ester with ethyl nitrite, as previously described [127]. SNAP and L-SNCEE were decomposed at 37ºC for 24 hours.

Plasmid Construction: The MTF-1 coding sequence was released from plasmid pCMV-MTF-1 by digestion with *NotI* and the resulting 5' and 3' ends were filled in using the Klenow fragment of *E.coli* DNA polymerase I. Plasmid pEGFP-C3 (Clontech) was cut with *Sac I* and the exonuclease activity of T4 polymerase was used to remove the resulting ends. The blunt-ended MTF-1 coding sequence was ligated into the prepared pEGFP-C3 vector using a Rapid Ligation Kit (Roche) resulting in pEGFP-MTF1-C3.

Cultured sheep pulmonary artery endothelial cells (SPAEC): SPAEC were cultured from sheep pulmonary arteries obtained from a nearby slaughterhouse as previously described [128]. The

SPAECs were grown in OptiMEM (GIBCO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in an atmosphere with 5% CO_2.

Cultured murine lung endothelial cells (MLEC)*:* The transgenic mice (MT $^{+/+}$ and MT $^{-/-}$) are existing colonies that were imported from Australia (courtesy of Drs. A. Michlaska and A. Choo). MLEC were isolated by modifications to established protocols using rat anti-mouse CD31 (i.e. PECAM) monoclonal antibody-coated magnetic beads and characterized by panels of endothelial cell-specific markers [25]. MLEC were grown in low-glucose DMEM, 20% FBS, 20 mM HEPES, 2 mM glutamine, 100U/mL penicillin, 100 µg/mL streptomycin and 200 µg.mL Endo Gro (VEC Technologies, Inc) at 37°C in an atmosphere with 5% CO_2.

Transgene and native protein expression: Expression of MT was quantified by Western blot, using commercially available monoclonal antibodies (E9; Dako) and modifications of established protocols [129]. Cells were harvested by trypsinizing, washed once in PBS (pH 7.4) and resuspended in lysis buffer (10 mM Tris, pH 7.5, 0.1% SDS, 0.1 mM EDTA, adjusted to 10 mM DTT just before use). The resuspended cells were disrupted by sonication and allowed to rest on ice for 10 minutes after which the resulting whole cell lysate was cleared by centrifugation at 12,000 x g for 10 minutes at 4°C. Protein concentrations in the lysates were measured using the Bio Rad Protein Assay. Approximately 30 µg of total protein was heated at 65°C for ten minutes in 1X Kimura Buffer (0.04 M Tris, pH 8.8, 1.6% SDS, 10% glycerol). Samples were then incubated at room temperature, in the dark, for thirty minutes in the presence of 0.1 M iodoacetamide (Sigma). Proteins were separated on a 16% Tricine gel (Novex, Invitrogen) and transferred to a nitrocellulose membrane. After transfer, the membrane was fixed in 2% glutaraldehyde (Sigma) for 5 minutes on ice before incubation with anti-metallothionein antibody (E9, Dako).

Levels of MTF-1 were also determined by Western blotting using the monoclonal antibody for MTF-1 kindly provided by Dr. Glen Andrews (University of Kansas). Whole cell lysates for determination of MTF-1 levels were obtained as above in the MT Western blotting protocol.

Nuclear and cytosolic extracts*:* Cellular extracts were prepared using established protocols [130; 131] based on modifications of the method of Dignam [132]. After treatment, cells were scraped from the flask in 1 mL ice-cold PBS and collected by centrifugation at 250 x g for 5 min at 4°C. Cell pellets were resuspended in 5 mL of cell lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF). Resuspended pellets were centrifuged at 1500 x g for 5 minutes at 4°C. The cells were resuspended in 2 times pcv (packed cell volume) of cell lysis buffer, allowed to swell on ice for 10 minutes and homogenized with 10 strokes of a Dounce homogenizer. Nuclei were collected by centrifugation at 3300 x g for 15 min at 4°C. The cytosolic supernatant was cleared by centrifugation at 75,000 x g for 1 hour at 4°C. The resulting supernatant was concentrated in a Microcon 3 microconcentrator by centrifugation at 14,000 x g for 1 hour at 4°C. The nuclear pellet was suspended, using 6 strokes of a Dounce homogenizer, in 3 volumes of nuclear extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 400 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and 25% glycerol). The nuclear suspension was stirred on ice for 30 min, and then centrifuged at 75,000 x g for 30 minutes at 4°C to clear the suspension. The supernatant was concentrated in a Microcon3 microconcentrator by centrifugation at 14,000 x g for 3 hours at 4°C. Protein concentrations were measured using the BioRad Protein Assay and samples were processed for Western blotting as described in MTF-1 blotting protocol above.

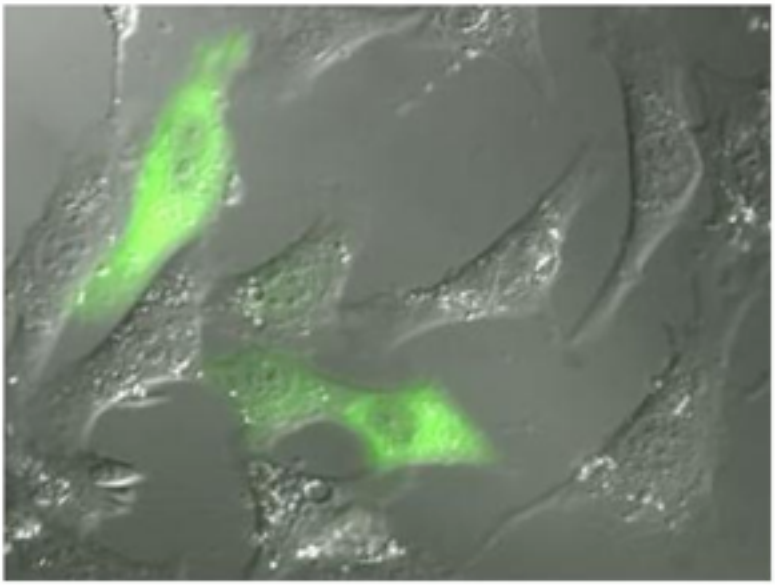
Determination of MTF-1 Localization: SPAEC or MLEC were plated in two-well chambers with coverslip bottoms (Labtek II). Cells were transfected with 50ng/well plasmid using Lipofectamine Plus (Gibco/BRL). Two-four hours after transfection, cells were treated with $ZnCl_2$ (200 μM), S-Nitroso-N-acetylpenicillamine (SNAP, 200 μM) or L-SNCEE (1 pM) for two hours. For fixation, cells were treated with 4% paraformaldehyde for 5 min on ice and imaged using a Nikon Diaphot inverted microscope. Images were collected with a Photometrics charge coupled device camera with MetaMorph software. Localization of pEGFP-MTF-1 was classified as nuclear, cytoplasmic, or both nuclear and cytoplasmic. Means and standard deviations were derived from three independent experiments, each sampling a minimum of 200 cells.

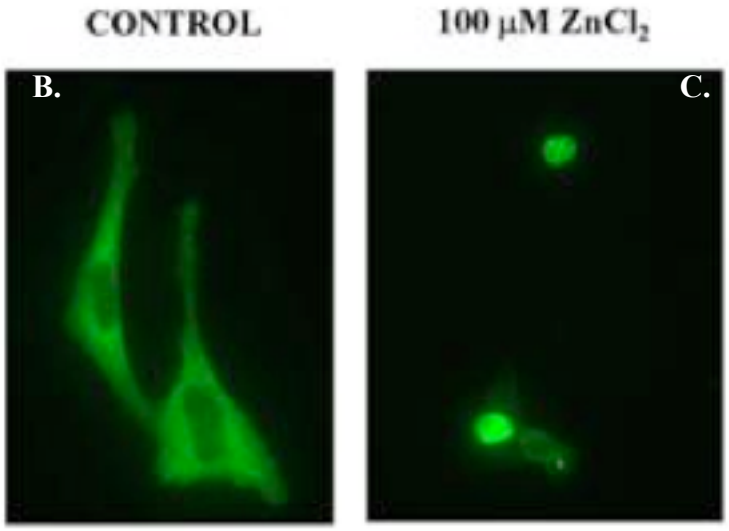
2.3.4 Results

2.3.4.1 EGFP-MTF-1 translocates to the nucleus in response to zinc or NO.

We generated a reporter molecule consisting of enhanced green fluorescent protein (EGFP) fused to the N- terminus of MTF-1 in an effort to investigate the effects of NO on the subcellular localization of MTF-1 in intact cells. SPAEC were transiently transfected with the EGFP-MTF-1 expression vector and examined by confocal microscopy 2 h after treatment with NO donors or zinc. Whereas native MTF-1 has been shown to be almost exclusively localized to the cytoplasm in serum-starved cells [131; 133], a considerable fraction of the protein is found in the nucleus under normal growth conditions [65; 133]. Consistent with these reports, EGFP-tagged MTF-1 was detected in both the cytoplasm and nucleus of untreated cells (Fig. 2.1).

A.





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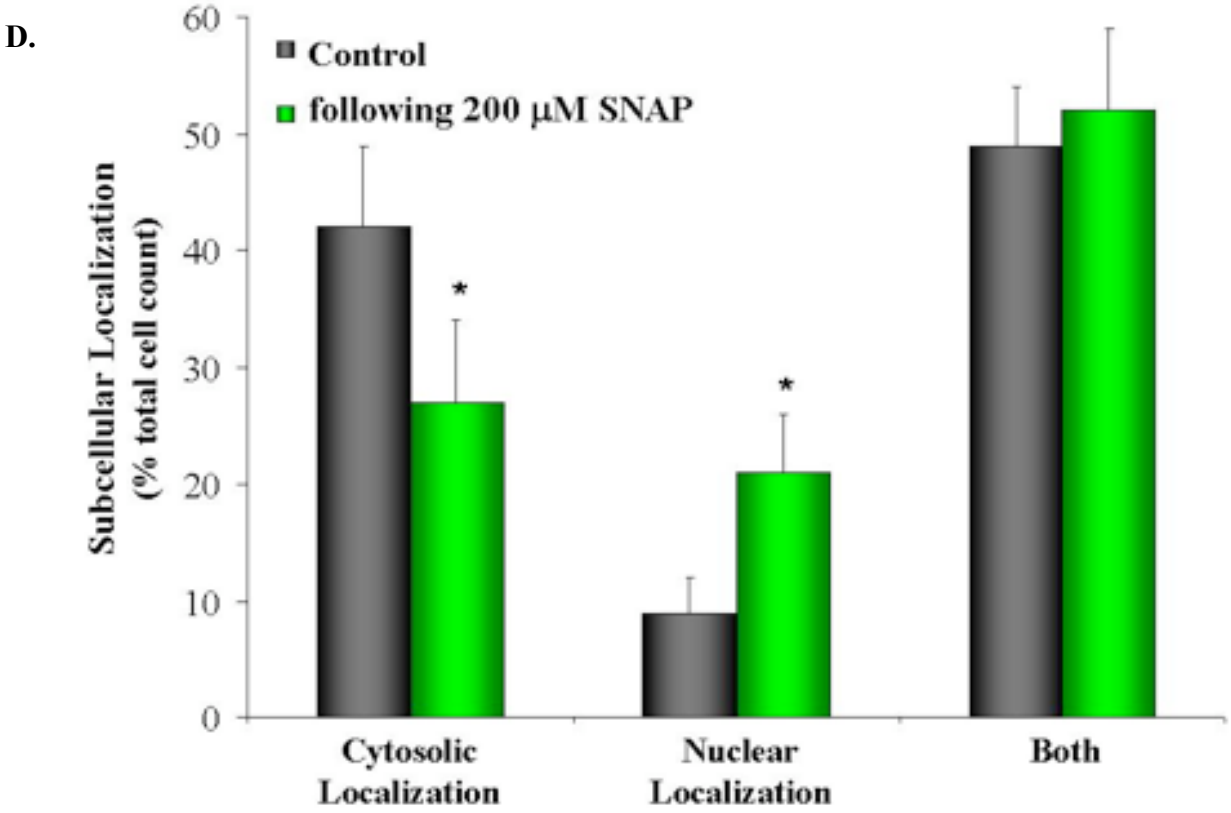


Figure 2-1 SPAEC transiently transfected with EGFP-MTF-1 chimera. Under normal conditions, the chimeric protein is found both in the cytosol and nucleus of the cells (panels 2.1A and 2.1B). Upon addition of 200 μM $ZnCl_2$, a significant portion of the chimeric protein migrates to the nucleus (panels 2.1C and 2.1D). After a 2 hr treatment with the nitric oxide donor SNAP (200 μM), the EGFP-MTF-1 chimera had migrated to the nucleus of a significant portion of the cells (panel 2.1D).

However, nuclear accumulation of pEGFP-MTF-1 was significantly increased in response to exogenously applied $ZnCl_2$ (200 μM, Fig. 2.1B and 2.1C). The NO donor, SNAP (200 μM) also caused significant nuclear translocation of EGFP-MTF-1 (Fig. 2.1D) though the effects were not as pronounced as those for zinc.

We performed Western blotting on nuclear extracts prepared from SPAEC using rabbit polyclonal anti-MTF-1 to confirm that native MTF-1 also responded to NO and found that 2 hr

exposure to either $ZnCl_2$ (200 μM) or SNAP (200 μM) caused increased nuclear localization of MTF-1 (Fig. 2.2).

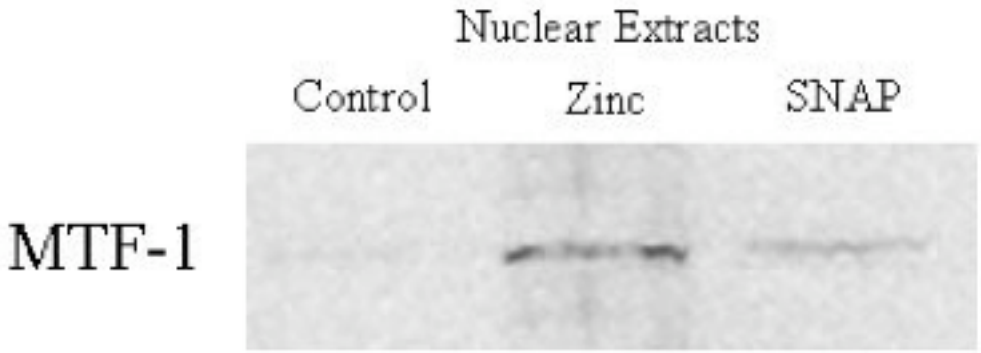


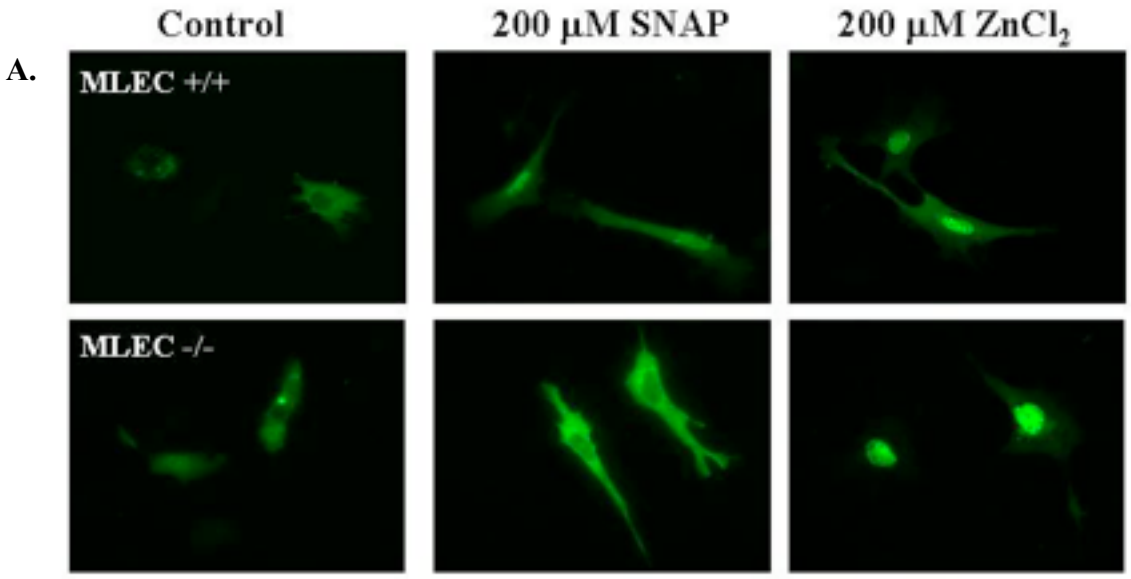
Figure 2-2 MTF-1 western blot of nuclear extracts from SPAEC. MTF-1 was detected using rabbit polyclonal anti-MTF-1 (kindly donated by G. Andrews) showing that a 2 hr exposure to either 200 μM $ZnCl_2$ or 200 μM SNAP caused increased nuclear localization of MTF-2. Western blotting of whole cell lysates showed that neither treatment affected changes in total cellular MTF-1 protein (data not shown).

Neither treatment affected changes in total cellular MTF-1 protein (data not shown).

2.3.4.2 NO-mediated nuclear translocation of pEGFP-MTF-1 is dependent on metallothionein

We and others have demonstrated NO-mediated increases in labile zinc in several cell types [62; 75; 134; 135]. Furthermore, the absence of a response in cells derived from MT null mice indicated that MT was the requisite target for NO-induced changes in intracellular zinc homeostasis [62; 75]. We transiently transfected pulmonary endothelial cells from MT +/+ and -/- mice with the EGFP-MTF-1 reporter construct to test the hypothesis that NO-induced

activation of MTF-1 was via zinc release from MT. In agreement with the data obtained in SPAEC (Fig. 1) MLEC from MT +/+ animals showed a significant increase in the nuclear localization of the pEGFP-MTF-1 chimera following 2 h treatment with the NO donor S-nitroso-N-acetyl penicillamine (SNAP, 200 μM, Fig. 2.3, upper panel). In contrast, SNAP did not cause a nuclear translocation of EGFP-MTF-1 in MLEC that were isolated from MT null mice (Fig. 2.3, lower panel) demonstrating a central role for MT in mediating these responses.



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B.

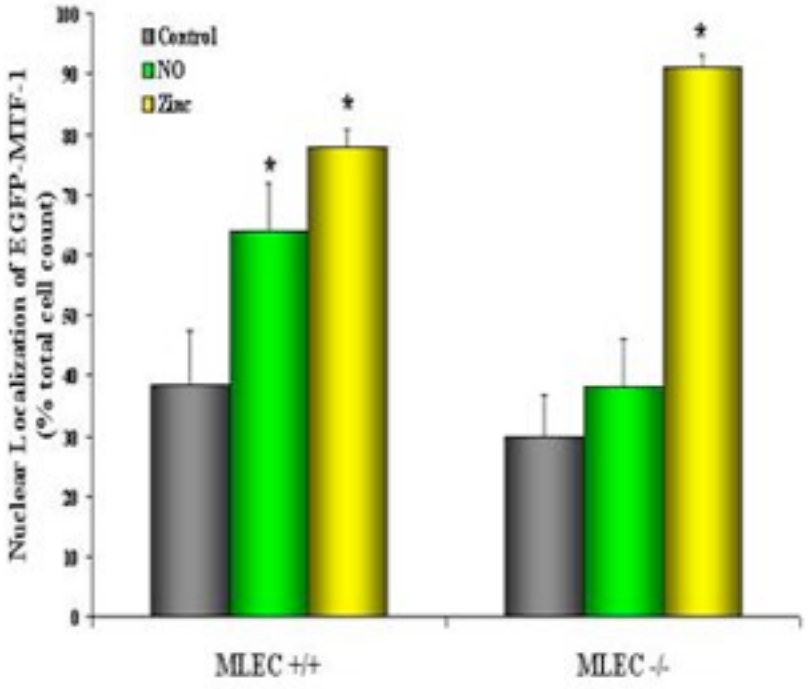

Figure 2-3 Wild-type and metallothionein null mouse pulmonary endothelial cells transiently transfected with the EGFP-MTF-1 chimera. In wild-type cells, exposure to both the nitric oxide donor SNAP (200 μM) and $ZnCl_2$ (200 μM) caused the fluorescent chimera to migrate to the nucleus of a significant number of the cells examines (2.3A, top panel and 2.3B). In contrast, only exposure to $ZnCl_2$ (200 μM) caused nuclear migration of the chimera in metallothionein null cels (2.3A, bottom panel and 2.3B)

2.3.4.3 NO induces MT protein expression.

MTF-1 binds to metal responsive elements present in the proximal promoter of the MT I and MT II genes in response to increases in intracellular zinc [136]. We hypothesized that the NO-induced changes in labile zinc would impact upon the activity of MTF-1 and, in this manner, affect changes in expression of MT protein. Western blotting showed that MT protein levels were increased in response to the NO-donor SNAP (200 μM, Fig. 2.4) and low concentrations of

S-nitroso-L-cysteine ethyl ester (L-SNCEE, Fig. 2.4). The increase in protein expression was apparent at 2 h, peaked at 8 h and had returned to baseline levels by 12 h.

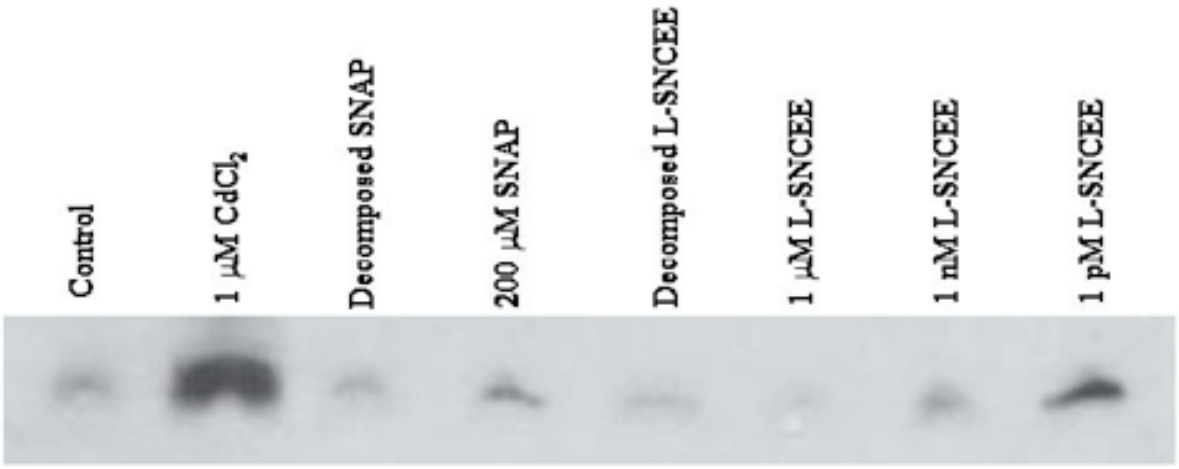

Figure 2-4 Metallothionein western blotting of SPAEC whole cell extracts exposed to NO donors. MT was detected using mouse monoclonal anti-metallothionein (Clone E9, Dako) showing that a 2 hr exposure to the nitric oxide donors SNAP (200 μM) and S-nitroso-cysteine ethyl ester (SNCEE) at low doses caused upregulation of metallothionein protein expression. MT protein expression was also upregulated in the presence of 1 μM $CdCl_2$ (positive control) and was not affected by decomposed nitric oxide donors.

2.3.5 Discussion

It is apparent that S-nitrosation of zinc sulfur clusters is an important component of NO signaling and that metallothionein is critical link between NO and intracellular zinc homeostasis [62; 75]. The present data demonstrates that the metal responsive transcription factor, MTF-1, is one cellular target for the MT-released zinc with a resultant impact upon gene expression.

2.3.5.1 Activation of MTF-1

The metallothionein genes are the best characterized of the target genes for MTF-1. Heavy metal exposure [64], reactive oxygen species [136] and hypoxia [137] have all been shown induce MT gene transcription through activation of MTF-1. While the mechanisms of activation are only partially understood, recent evidence [74; 138; 139] supports the hypothesis that metals and oxidants activate MTF-1 by causing a redistribution of zinc in the cell [67; 72]. MTF-1 is regulated by micromolar concentrations of zinc [140], and itself may function as a sensor of cytoplasmic zinc via a subset of zinc fingers with lower binding affinities for the metal [67; 141; 142]. Zinc binding would lead to an allosteric change in MTF-1 causing exposure of zinc fingers involved in DNA binding [67]. In support of this model, zinc fingers 1, 5 and 6 appear to play an essential role in metal-induced recruitment of MTF-1 to the MT-1 promoter and formation of a stable MTF-1-chromatin complex [143].

We have shown that: 1) NO increases labile zinc in pulmonary endothelial cells [62]; and 2) causes increases in the nuclear localization of MTF-1 (Figs. 2.1-2.3). The absence of these NO-induced effects on zinc homeostasis and MTF-1 localization in cells derived from MT-null mice revealed that MT was central to both processes. Recent data obtained using a cell-free, MTF-1-dependent transcription system [74], supported the hypothesis that metallothionein is critical to the modulation of MTF-1 activity. Cadmium, copper or hydrogen peroxide induced transcription only in the presence of zinc-saturated MT. Furthermore, the apo-protein, thionein, inhibited the activation of MTF-1, presumably by sequestering zinc. Our data suggests that the mechanism of MTF-1 activation by metals released from MT is also relevant in vivo.

The nuclear localization of MTF-1 appears to be essential, but not necessarily sufficient, for the transcriptional activation in response to zinc [133]. It is increasingly apparent that

phosphorylation *via* multiple kinases including protein kinase C (PKC), c-Jun N-terminal kinase (JNK) and tyrosine kinase, is involved in the metal-dependent transactivation of MTF-1 [144; 145; 146]. Multiple MAP kinase signaling pathways, including JNK have also been implicated in the cellular responses to NO related species [147; 148] *via* S-nitrosation of critical cysteine residues [149]. While these reports suggest that NO could directly affect the phosphorylation of MTF-1, this is not sufficient to induce translocation of MTF-1 in MT-null cells.

2.3.5.2 Induction of MT by NO

NOS-derived NO and NO donors were both recently shown to increase MT mRNA expression in bovine aortic endothelial cells [75]. In support of these findings our data confirms that MT protein is increased in response to NO and provides the first direct evidence for an effect of NO on MTF-1. Furthermore, physiologically relevant increases in NO have been shown to induce glutathione (GSH) synthesis in vascular endothelial cells, in part through increased expression of gamma-glutamylcysteine synthase (γGCS) (78). MTF-1 is a crucial transcriptional regulator for basal expression of γGCS [70] and while the response was independent of cGMP [150; 151], the mediators involved in the NO-dependent signaling for γGCS remain unclear. Conversely, iNOS inhibition decreased MT I and MT II in brain and liver [152]. It is not clear whether the discrepant results are tissue specific, are due to non-specific effects of the inhibitor, or reflect concentration dependent effects of NO on transcriptional activation.

2.3.5.3 Interactions of MTF-1 and NO

The zinc finger structure is the most prevalent DNA binding motif of transcription factors [153]. NO has been shown to disrupt the zinc fingers of LAC9 [59], Sp1 and EGR-1 [134] causing reversible inactivation of gene transcription. In contrast to the zinc clusters of MT, the

zinc fingers of MTF-1 do not appear to be targets for NO, in that disruption of the zinc thiolate bonds would actually be expected to weaken the DNA binding affinity of MTF-1 [67]. In contrast, we observed an activation of MTF-1 in response to NO, as evidenced by the increase in nuclear localization of MTF-1 and induction of MT protein. Such specificity of targets has been predicted to be secondary to thiol nucleophilicity (e.g. pKa) and has been reported to be affected by allosteric factors, hydrophobicity, subcellular localization and complex aspects of the three dimensional structure of the protein [91].

2.3.5.4 Perspective

NO may either promote or inhibit apoptosis [154; 155]. In general, if a cell is depleted of GSH and is undergoing oxidative stress, then exposure to large amounts of NO invariably leads to cell death. In contrast, in a cell with a more favorable redox status, low doses of NO tend to be anti-apoptotic. This phenomenon was originally noted in cultured endothelial cells that became resistant to LPS-induced apoptosis when synthesizing NO after direct gene transfer of human inducible nitric oxide synthase (iNOS) [23; 156].

We previously reported that S-nitrosation of Zn thiolate clusters in MT is a critical component of cellular redox sensitivity linking NO to Zn homeostasis in pulmonary endothelial cells [61; 62]. We now show that the resultant NO-induced changes in labile zinc impact upon the activity of the Zn-sensitive transcription factor MTF-1. While the physiological implications of this novel pathway have yet to be identified, NO-induced activation of MTF-1 and increased expression of protective genes may contribute to the observed anti-apoptotic effects of NO and protection from oxidative stress.

2.3.6 Acknowledgements

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3.0 CHAPTER 3: DETECTION OF S-NITROSOTHIOLS IN PULMONARY ENDOTHELIAL CELLS

In Preparation

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3.1 INTRODUCTION

We expanded upon our study of NO-mediated gene regulation by examining the implications of S-nitrosation as an emerging cyclic GMP-independent, post-translational NO modification that may form a unique signaling pathway [81; 91; 93; 157; 158; 159]. We used a fluorescent modification of the biotin switch technique [77; 116] in combination with proteomic techniques to examine the S-nitrosation status of metallothionein in sheep pulmonary artery endothelial cells

exposed to the NO-donor L-S-nitrosocysteine ethyl ester (L-SNCEE). Previous work in our laboratory had suggested that the effect of NO on zinc homeostasis in endothelial cells might be dependent upon S-nitrosation of the cysteine residues in metallothionein [60; 61; 62]. We examined this hypothesis in greater detail using our modified biotin switch technique and were able to demonstrate that metallothionein is in fact nitrosated in sheep pulmonary artery endothelial cells.

We then expanded our study of S-nitrosation in endothelial cells to include five types of cultured endothelial cells isolated from four different species; human, mouse, rat and sheep. Using our fluorescent S-nitrosothiol detection technique in combination with proteomic analyses we were able to identify potential protein targets of S-nitrosation from several categories of cellular proteins including cytoskeletal, chaperone, and glycolytic proteins. We also examined the effect of increased intracellular nitric oxide production via an adenoviral vector encoding the cDNA for human iNOS.

3.2 MANUSCRIPT AS PREPARED FOR SUBMISSION

3.2.1 Abstract

S-nitrosation is emerging as a potentially important post-translational signaling pathway. To study nitric oxide-mediated S-nitrosation in endothelial cells we modified the S-nitrosoprotein detection method known as the biotin switch to allow us to fluorescently tag and detect S-nitrosated proteins directly using two dimensional gel electrophoresis and mass peptide

fingerprinting. We used the fluorescent modification of the biotin switch assay to identify S-nitrosated metallothionein in endothelial cells. In addition we analyzed S-nitrosation of the proteome in human, mouse, rat, and sheep endothelial cells exposed to both exogenous and endogenous nitric oxide. We identified several novel targets of S-nitrosation in endothelial cells including members of the protein disulfide isomerase family, heat shock organizing protein 1, and aldolase A. We have developed a fluorescence-based proteomic screening method to identify S-nitrosoproteins in endothelial cells, which will allow us to continue to study nitric oxide-mediated signaling pathways in endothelial cells.

3.2.2 Keywords

S-nitrosation

Nitric Oxide

Endothelium

Endothelial Cells

S-nitrosoalbumin

S-nitrosylation

Biotin Switch

3.2.3 Introduction

Nitric oxide (NO) is an important modulator of cellular function. It is a small, easily diffusible molecule known to affect many cellular functions including smooth muscle cell relaxation in the vasculature, and neurotransmission. It also contributes to the defense

mechanisms of the immune system. NO is a reactive molecule due to its paramagnetic nature. It can react with cellular metals to form metal-nitrosyl compounds, with oxygen to form nitrite and nitrate, with superoxide to form peroxynitrite, and with cellular nucleophiles [34]. Most of the focus on NO-mediated signaling involves the metal dependent activation of the cyclic guanosine monophosphate (cGMP) signaling pathway. In this pathway NO binds to the active site heme of guanylyl cyclase and leads to an increase in formation of cGMP [160]. However, in recent studies a new, cGMP-independent signaling pathway involving nitrosation of protein thiols has emerged.

Thiols are among the most numerous cellular nucleophiles and recent studies have demonstrated that S-nitrosothiols may play an important role in cellular signaling [81; 159; 161]. In this study we have focused on a particular aspect of NO's interaction with cellular thiols, namely S-nitrosation. S-nitrosation reactions involve an attack by a nucleophilic thiol group on a nitrosonium (NO+) equivalent [161]. In the cellular milieu, certain molecular species can be thought of as "carriers" of nitrosonium, including metal-nitrosyls, nitrite and peroxynitrite, and perhaps most importantly, other S-nitrosothiols [93]. Transfer of nitrosonium equivalents between an existing S-nitrosothiol and the target thiol is referred to as transnitrosation [161].

Emerging evidence has demonstrated that S-nitrosation of proteins can affect their function, and in some cases their sub-cellular distribution. In most cases this phenomenon can be traced to post-translational modification of a specific cysteine thiol or thiols in the protein [159]. In addition, it has been postulated that there are enzymes that may remove this NO-based modification in a specific manner [84; 162; 163]. When all of the studies are considered, S-nitrosation seems to be an emerging post-translational signaling mechanism somewhat analogous to protein phosphorylation.

In addition, dysregulation of or changes to S-nitrosative signaling in cells has been linked to a number of diseases. Decreased levels of S-nitrosothiols have been found in the airway lining fluid in asthma while S-nitrosothiol levels are elevated in the synovial fluid of the joints in arthritis [30]. Studies have also identified increases in S-nitrosoalbumin in plasma from patients with pre-eclampsia [164; 165]. Finally, elevated levels of S-nitrosohemoglobin have been found in patients suffering from pulmonary hypertension [30]. When combined with the emerging evidence for NO participation in specific signaling pathways, these links to disease highlight the importance of further study of S-nitrosothiol based signaling events in cells.

Detecting S-nitrosothiols in biological systems can be difficult. There are a variety of measurement systems including chemical and flash-photolysis that can be used in conjunction with a chemiluminescent detection techniques [107]. However, chemical detection systems can produce artifacts when used to study complex systems such as whole cell lysates since other forms of NO are detected in addition to S-nitrosothiols. Flash-photolysis chemiluminescence is a very sensitive technique, but it is generally used to detect either total S-nitrosothiols in a sample, or the S-nitrosation of a particular, purified protein. This technique does not lend itself easily for use in proteomic methods. Recently, Jaffrey and his colleagues have developed a technique to identify S-nitrosothiols in whole cell lysates known as the biotin-switch method [77; 116]. The original version of this method replaces the NO moiety of the S-nitrosothiol with a biotin moiety (*N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide–biotin-HPDP) that can be detected on an immunoblot using an anti-biotin antibody or an appropriately labeled streptavidin molecule. We have modified this method to allow it to be used more efficiently in proteomic techniques. We increased the number of free thiol blocking steps to reduce background and increased the ascorbate concentration and incubation time to increase sensitivity in S-nitrosothiol

detection. In addition we have replaced the biotin moiety with a fluorescent label in order to allow S-nitrosated proteins to be detected directly using two-dimensional gel electrophoresis.

Using our modified method, we examined the S-nitrosation of the proteome in endothelial cells from several species (human, rat, sheep and mouse) in the presence and absence of both exogenous (L-SNCEE and S-nitrosoalbumin) and endogenous (an adenoviral vector expressing iNOS) NO donors.

3.2.4 Materials and Methods

3.2.4.1 Materials

AdiNOS was a gift from Tim Billiar at the University of Pittsburgh. Cell culture media and supplements were obtained from Invitrogen except where noted in the text. Heparin, HEPES, EDTA, neocuproine, MMTS, CHAPS, trifluoroacetic acid and ammonium bicarbonate were obtained from Sigma. Two-dimensional gel electrophoresis (2DGE) grade urea was obtained from Invitrogen. Ascorbate was obtained from Aldrich, while acetonitrile was from Pierce and α-cyano-4-hydroxycinnamic acid was from Fluka.

3.2.4.2 Cell culture and isolation

Human Pulmonary Artery Endothelial Cells (HPAEC) were a gift from Aaron Barchowsky (University of Pittsburgh). Rat Pulmonary Artery Endothelial Cells (RPAEC) were a gift from Troy Stevens (University of Alabama, Birmingham). Rat microvascular endothelial cells were purchased from VEC Technologies, Renssalaer, NY. RPAEC were cultured in Dulbeccos' Modified Eagle Medium (DMEM) with 10% FBS, 100 U/mL penicillin, and 100

μg/mL streptomycin at 37°C in an atmosphere of 5% CO_2. RMVEC were cultured in flasks precoated with sterile 1% gelatin in Hank's Buffered Saline Solution (HBSS, Invitrogen) in MCDB-31 complete medium (VEC Technologies) containing DMEM supplemented with 5% FBS, endothelial cell growth factor, heparin, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in an atmosphere of 5% CO_2. Sheep Pulmonary Artery Endothelial Cells (SPAEC) were cultured from sheep pulmonary arteries obtained from a local slaughterhouse as previously described [128] and grown in OptiMEM (Invitrogen) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in an atmosphere with 5% CO_2. Mouse lung endothelial cells were isolated by modifications of a previous protocol [25]. Briefly, mouse lungs were perfused and rinsed with a solution of HBSS with calcium and magnesium and 10U/mL heparin. Lungs were minced and digested with collagenase (Type I Sigma, 100 μg/mL in HBSS with calcium and magnesium) for 25 minutes with occasional agitation. The resulting digest was filtered 2X through 100 μm cell strainers and 1X through 75 μm cell strainer and centrifuged (800 rpm, 8 minutes, 4°C) to pellet cells. Cells were incubated in 1X binding buffer (1.5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 140 mM NaCl, 0.2 mM EDTA, 0.5% BSA) with sheep anti-rat Dynabeads (Invitrogen) that had been coated overnight with a rat anti-mouse monoclonal antibody to platelet endothelial cell adhesion molecule-1 (PECAM-1, BD Biosciences) according to manufacturer's instructions for 1 hour at 4°C with constant agitation. Cells were purified magnetically according to manufacturer's instruction (Dynal, Invitrogen) by rinsing 5 times in cell culture medium using the appropriate magnet for isolation. After washes, cells attached to beads were plated in 0.1 mg/mLcollagen/0.1% gelatin (both from Sigma) coated culture plates in MLEC medium [OptiMEM (Invitrogen), with 10% FBS, 100 U/mL penicillin, 100 μg/mL

streptomycin, EndoGro (VEC Technologies), 2 mM L-glutamine, 1X non-essential amino acids (Invitrogen), 55 μM β-mercaptoethanol, and 10 U/mL heparin) in an atmosphere with 5% CO_2.

3.2.4.3 Synthesis of S-nitrosoalbumin

S-nitrosoalbumin was synthesized and characterized as previously described [83].

3.2.4.4 Treatment and harvest of endothelial cells for S-nitrosothiol detection

L-SNCEE was synthesized immediately before use as previously described [127]. Endothelial cells were exposed to 100 μM L-SNCEE in full growth media at 37°C for 30 minutes. Endothelial cells were exposed to S-nitrosoalbumin in HBSS with calcium and magnesium at 37°C for 30 minutes. Cells were harvested immediately by trypsinizing and cell pellets were washed 1X in HBSS before being resuspended in HEN buffer. Cells were sonicated (6 X 1 second pulses at 50% maximum input, Sonics and Materials Vibracell VC-30) and lysates were cleared by centrifugation at 10,000 x g at 4°C for 10 minutes. Protein concentration was measured using the Bio Rad Protein Assay and supernatants were stored at -80°C until used in the S-nitrosoprotein detection assay.

3.2.4.5 Metallothionein western blot

SPAEC were infected with a 500:1 multiplicity of infection (MOI) of AdMT and whole cell lysates were harvested at 24 hours post-infection by trypsinizing and sonicating cells (6 X 1 second pulses at 50% maximum input, Sonics and Materials Vibracell VC-30) in HEN buffer

(see composition below). Samples were processed for S-nitrosothiol detection as below and 2D gel electrophoresis was performed as below using pH 3-10NL ZOOM strips (Invitrogen) and 4-20% Tris-Glycine ZOOM IPGRunner gels. Samples were transferred to nitrocellulose membrane, the membrane was fixed in 2% glutaraldehyde for 5 minutes at 4°C, and MT was detected using a primary mouse anti-horse MT antibody (Clone E3, Dako) and a rat anti-mouse Alexa 488 secondary antibody (Invitrogen) [166]. Fluorescent images of membranes were obtained using a Typhoon imaging system (GE Lifesciences).

3.2.4.6 Detection of S-nitrosothiols and two-dimensional gel electrophoresis

S-nitrosothiols were detected in cellular lysates using the biotin-switch method with the following modifications [77; 116]. Protein samples were resuspended at a concentration of 0.8 µg/µL in 100 uL total volume HEN buffer (250 mM HEPES, pH 8.0, 1 mM EDTA, 0.1 mM neocuproine). Proteins were incubated with four volumes of MMTS blocking buffer (HEN buffer with 2.5% SDS and 20 mM MMTS) at 50°C in the dark for twenty minutes with frequent vortexing. Protein was precipitated by addition of two volumes of ice-cold acetone and incubation at -20°C for 20 minutes followed by centrifugation at 12,000 x g at 4°C for 15 minutes. Precipitated protein was washed with ice-cold acetone and centrifuged at 12,000 x g at 4°C for 5 minutes. Protein was then resuspended in 100 µL HEN buffer and MMTS blocking was repeated as described above. After the second round of blocking, protein was precipitated and washed as described above. Each sample was resuspended in HENS buffer (HEN buffer, pH 8.0 adjusted to 1% SDS) and ascorbate was added to a final concentration of 30 mM. Alexa-547 labeled maleimide (Invitrogen) was added to each sample to a final concentration of 2 mM and samples were incubated at room temperature in the dark for two hours with gentle rocking. The

labeling reaction was stopped by running each sample through the Plus One 2D Gel Sample Clean Up Kit according to manufacturer's instructions (GE Lifesciences). Samples were resuspended in 1X IEF sample buffer (8M urea, 2% CHAPS, 0.002% bromophenol blue), applied to ZOOM IPGRunner IEF strips (Invitrogen), and strips were allowed to hydrate overnight. ZOOM IPGRunner IEF strips were focused according to manufacturer's suggestions (ZOOM IPGRunner, Invitrogen) and were run on 4-20% Tris-Glycine ZOOM Gels (Invitrogen) for second dimension. Fluorescent images of S-nitrosated proteins were obtained using a Typhoon imaging system (GE Lifesciences) and gels were stained with colloidal Coomassie blue for 18 hours (BioRad Bio-Safe). Protein spots of interest were picked from coomassie stained gels and prepared for mass peptide fingerprinting.

3.2.4.7 Preparation of proteins and mass peptide fingerprinting

Gel pieces containing proteins of interest were prepared for peptide mass fingerprinting as previously described [167]. Briefly, gel pieces were dehydrated at room temperature for 15 minutes in 2 x 100μL washes in 50% methanol/25 mM NH_4HCO_3. This step was followed by a 15 minute room temperature wash in 50% acetonitrile/ 25 mM NH_4HCO_3 (100 μL per sample). Gel pieces were incubated in 50 μL 100% Acetonitrile for 5 minutes at room temperature and then dried to completion at 65°C. Trypsin digestion was performed using 10 μL of 20 ng/μL trypsin (Promega Trypsin Gold) in 25 mM NH_4HCO_3 at 37°C for 18 hours. Digests were removed to a new tube, and peptides were eluted with 30 μL 1% trifluoroacetic acid (15 minutes at room temperature) followed by an elution step with 30 μL 50% acetonitrile/ 1% trifluoroacetic acid (15 minutes at room temperature). Supernatants containing peptides were evaporated to

dryness in a SpeedVac at room temperature. The dried peptides were resuspended in 50% acetonitrile/ 0.3% trifluoroacetic acid and mixed with an equal volume of saturated α-cyano-4-hydroxycinnamic acid. Duplicate 0.75 μL aliquots were spotted on a 192-well matrix-assisted laser desorption ionization (MALDI) plate (Applied Biosystems). MALDI-time of flight (MALDI-TOF) and MALDI-TOF-TOF analyses were performed using an ABI 4700 mass spectrometer at the University of Pittsburgh Integrated Genomics and Proteomics Core Facility. MALDI-TOF analysis was performed in the reflectron mode. Peak lists were analyzed using an in house version of Mascot (Matrix Science).

3.2.4.8 Infection of sheep pulmonary artery endothelial cells with AdiNOS and measurement of nitric oxide production

SPAEC were infected with a 1000:1 MOI of an adenovirus encoding inducible nitric oxide synthase (AdiNOS). Uninfected and infected SPAEC (SPAEC and SPAEC.iNOS respectively) were cultured in the presence and absence of the nitric oxide synthase inhibitor L-NAME (1 mM) for 72 hours post-infection. Nitrate accumulation in the culture supernatant was measured using the Greiss reaction, as previously described [168; 169]. Absorbance was measured at 550 nm, and nitrite was quantified using $NaNO_2$ as a standard.

3.2.5 Results

3.2.5.1 Detection of S-nitrosated metallothionein

Previous evidence from our laboratory and others indicates that the low molecular weight zinc-binding protein metallothionein (MT) may play a role in NO-mediated signaling [60; 61; 62]. Previous studies in our laboratory demonstrated that exposure of a fluorescence resonance energy transfer based-MT reporter to either endogenous or exogenous NO resulted in release of zinc, presumably via S-nitrosation its cysteine thiols [60; 61]. We decided to use the S-nitrosation assay that we developed to examine the S-nitrosation state of MT in SPAEC. We used an adenoviral vector expressing the cDNA for human metallothionein IIa (AdMT) in order to increase expression of MT in SPAEC in hopes of being able to reach a level that would allow protein identification via MS analysis. We first labeled the S-nitrosoproteins present in SPAEC lysates using our S-nitrosoprotein detection protocol. Labeled samples were used in 2DGE in combination with western blotting for MT in order to determine whether MT is nitrosated in SPAEC (Figure 3.1).

In the overlay of the fluorescently labeled S-nitrosoproteins (Figure 3.1A; in red) and the MT immunoblot using a fluorescently labeled secondary antibody (Figure 3.1A; in green) it appears that MT is in fact S-nitrosated in SPAEC. This becomes more apparent when the S-nitrosoproteins and MT immunoblot are viewed in separate images (Figure 3.1B and C, respectively). Protein spots were picked from the same location on a gel run under identical conditions and stained with Coomassie for MS protein identification (Figure 3.1D). Unfortunately, the protein concentration was not high enough in the gel pieces to positively identify MT. However the primary antibody used in this study is highly specific for MT [166].

This leads us to believe that MT is S-nitrosated in SPAEC based upon the results of the S-nitrosoprotein detection assay performed in tandem with a MT immunoblot.

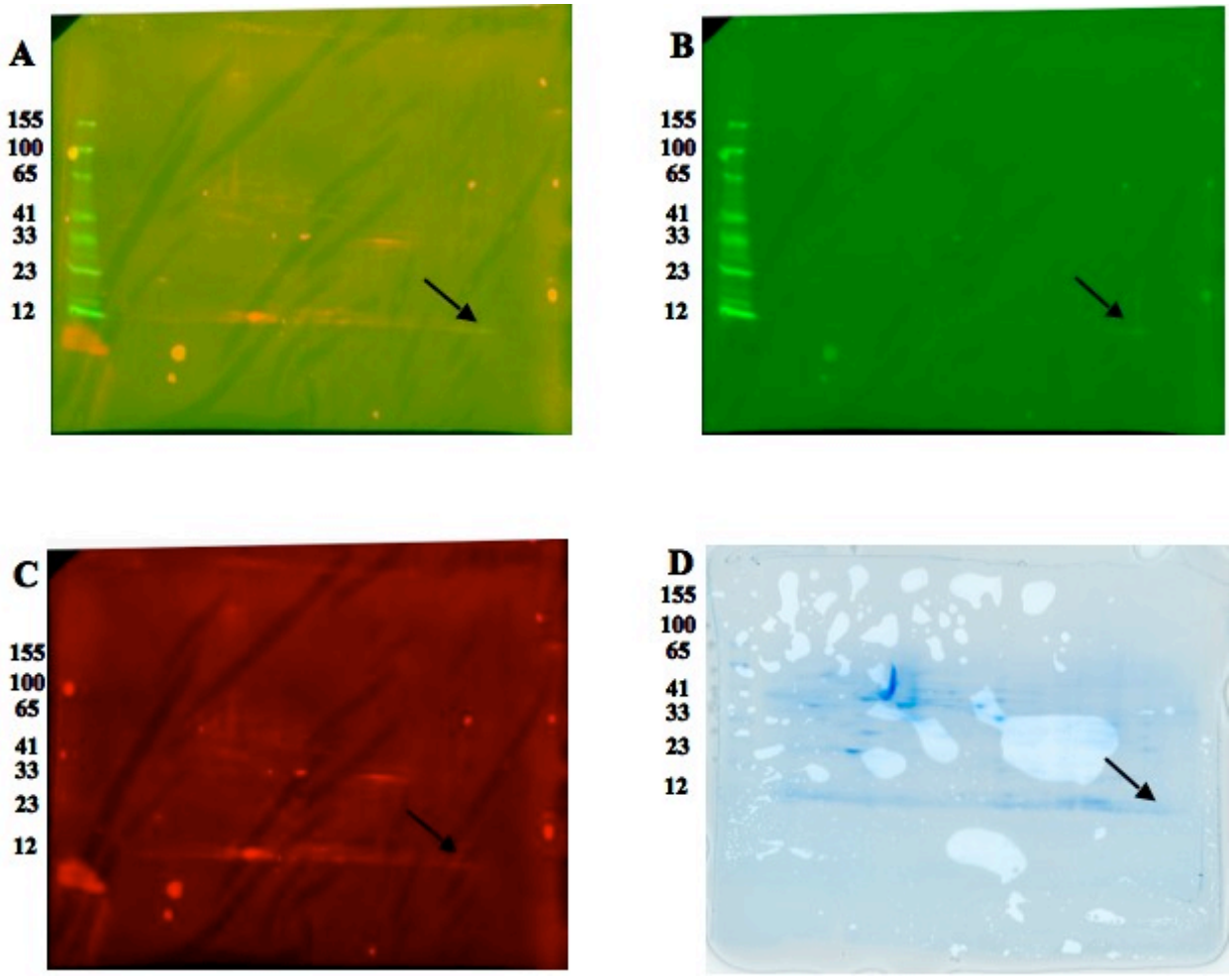

Figure 3-1 Detection of S-nitrosated metallothionein by S-nitrosation assay in combination with immunoblotting. SPAEC were infected with an adenovirus expressing the cDNA for human metallothionein IIa and cell lysates were harvested 24 hours post-infection. S-nitrosated proteins were labeled using an Alexa-647 maleimide in the S-nitrosoprotein detection assay (red). Metallothionein (MT) was detected using a primary anti-mouse antibody against MT and an Alexa-488 labeled secondary antibody (green). A) Overlaid image of both Alexa-647 labeled S-nitrosated protein and Alexa-488 labeled secondary antibody indicating the location of MT on membrane (arrow). B) Alexa-488 channel of image indication location of MT on membrane (arrow). C) Alexa-647 channel of image indicating presence of S-nitrosated proteins on membrane. S-nitrosated protein is present in

the same area where MT was detected (arrow). D) Coomassie stained gel image used to pick spots (from region indicated with arrow) for MS identification of MT.

3.2.5.2 S-nitrosation in rat pulmonary artery endothelial cells

We examined the formation of S-nitrosated proteins in rat pulmonary artery endothelial cells (RPAEC) exposed to two different NO donors. The first, L-SNCEE is a cell-permeant nitrosating agent [127]. The second, S-nitrosoalbumin, is a nitrosating agent that can participate in trans-nitrosation reactions [42; 83; 170; 171]. We exposed RPAEC to both donors and then used our modification of the biotin-switch assay in combination with two-dimensional electrophoresis and mass spectrometry to identify S-nitrosoproteins (Figure 3.2; Table 3.1 and 3.2).

In order to maximize resolution in our experiments we chose to use a miniature two-dimensional gel electrophoresis (2DGE) format (ZOOM IPGRunner, Invitrogen). The small size of the gels allowed us to ensure detection of S-nitrosated proteins given the relatively small amounts of protein harvested from cultured endothelial cells (in the μg range vs. the mg range necessary to use traditional large format [2DGE]) [172]. In order to verify consistency between gel runs using the miniature gel format, the experiment with RPAEC was repeated in triplicate. The gels shown are representative of each individual experiment, while the proteins identified are a compilation of positive identifications from all experiments (Figure 3.2; Table 3.1 and 3.2).

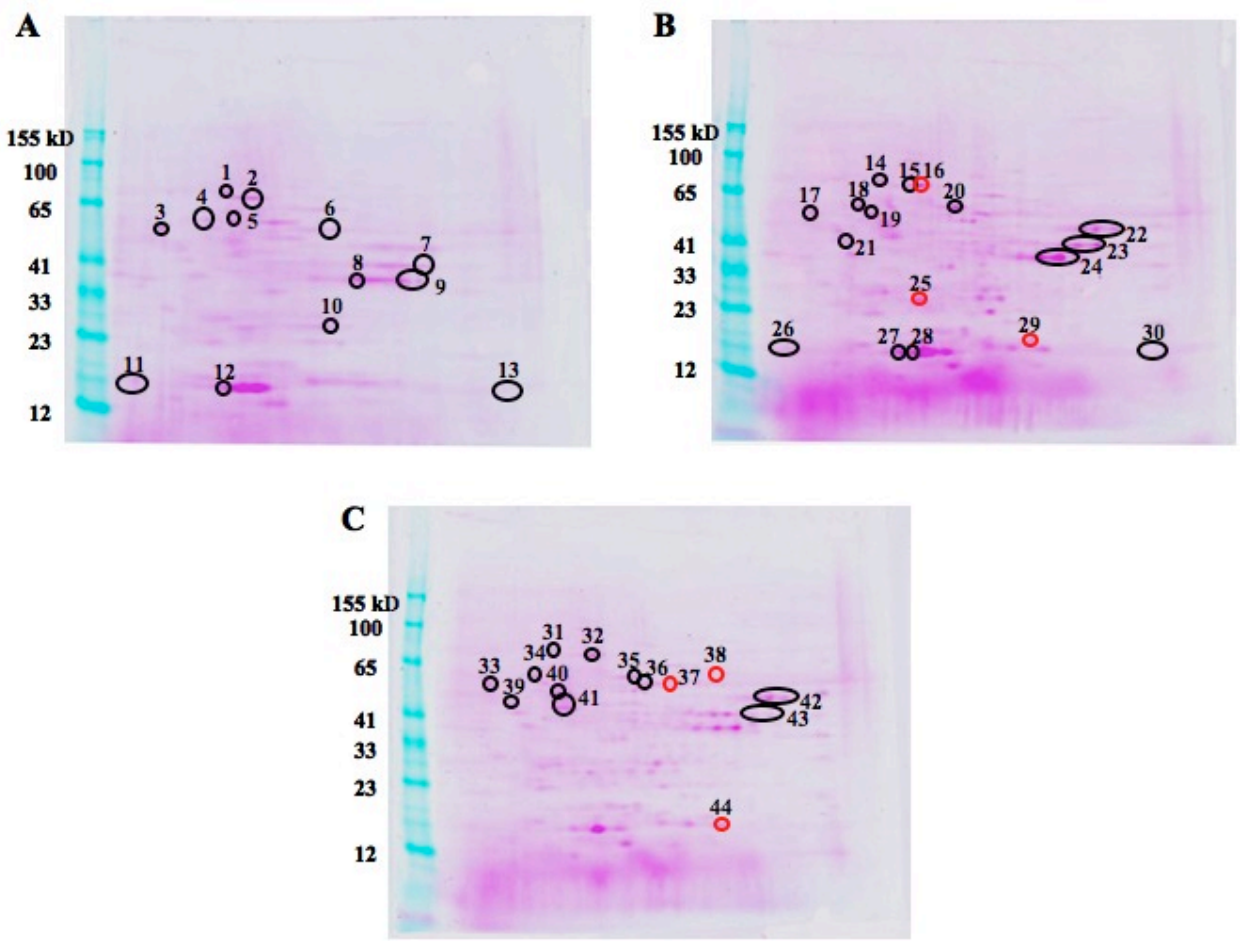

Figure 3-2 S-nitrosothiol detection in rat pulmonary artery endothelial cells (RPAEC). RPAEC were exposed to NO donor for 30 minutes as described in materials and methods. Cell lysates were used in the S-nitrosothiol detection assay in combination with 2DGE. Numbered spots represent S-nitrosated proteins identified by MS analysis. Numbers coordinate with Gel Position category in Tables 3.1 and 3.2 A) Untreated RPAEC; B) RPAEC exposed to 200 μM S-nitrosoalbumin; C) RPAEC exposed to 100 μM L-SNCEE

Table 3-1 Proteins not previously known to be S-nitrosated as identified by MS analysis. S-nitrosated proteins identified in RPAEC. Gel position identifiers indicate protein position on gels in Figure 3.1.

Gel Position	Protein	MW	PI	Conditions	Accession Number	Function
B16; C37	ERp60 protein (PDI-like)	63.0	6.4	SNO-Albumin and SNCEE	gi|3688521	Chaperone
B29	Expressed in non-metastatic cells 2	17.3	6.92	SNO-Albumin	gi|55778652	Signaling
B25	Thiol-specific antioxidant protein	24.8	5.64	SNO-Albumin	gi|3688521	Cytoprotective
C38	HOP1	63.0	6.40	SNCEE	gi|38181876	Chaperone
C44	Ribosomal protein large P2	17.1	4.46	SNCEE	gi|71795613	Protein synthesis
A2; B15; C32	Heat shock protein 8	7.1	5.37	All	gi|31981690	Chaperone
A6; B20; C35; C36	PDI associated 3	57.0	5.88	All	gi|38382858	Chaperone
A12; B27	B-galactoside-binding lectin	15.1	5.14	All	gi|9845261	Cytoskeleton
A11; B26	Non-muscle myosin alkali light chain	17.1	4.46	All	gi|998520	Cytoskeleton
A13; B30	Profilin	15.1	8.46	All	gi|1628436	Cytoskeleton
C39	Reticulocalbin	37.9	4.72	All	gi|53734248	ER Protein
A7; B23; C43	Aldolase A	39.6	8.31	All	gi|202837	Glycolysis
A10	Phoshopglycerate mutase 1	28.9	6.67	All	gi|76779285	Glycolysis
A4; B18; C34	Mixture of Iodothyronine 5' monodeiodinase And Prolyl 4-hydoroxylase	54.3 57.2	4.87 4.82	All	gi|202549 gi|38197382	Hormone Synthesis Collagen Synthesis
B22; C42	Serpin H1	46.6	8.88	All	gi|55824765	Signaling

Table 3-2 Proteins known to be targets for s-nitrosation as identified by MS analysis in this study. S-nitrosated proteins that have previously been identified as targets of S-nitrosation in other studies as identified in RPAEC exposed to NO donors are represented in the table. Asterisks indicate proteins identified as targets of S-nitrosation in previous studies in endothelial cells.

Gel Position	Protein	MW	PI	Conditions	Accession Number	Function	Known S-NO Modified
A1; B14; C31	Heat shock protein 70	72.4	5.07	All	gi|38303969	Chaperone	Yes [173; 174]
A5; B19	Vimentin	53.7	5.06	All	gi|57480	Cytoskeleton	*Yes [175]
C40; C41	Gamma actin	59.0	5.67	All	gi|109492380	Cytoskeleton	*Yes [77; 159; 176]
A3; B17; C33	Calreticulin	48.1	4.33	All	gi|38565977	Chaperone	Yes [173]
A9; B24	GAPDH	36.0	7.63	All	gi|56611127	Glycolysis	*Yes [77; 159; 175; 176; 177; 178]
B28	Eukaryotic translation initiation factor 5A2	17.1	5.38	All	gi|76827038	Protein Synthesis	Yes [92; 179]
A8	hnRNP A/B	36.3	6.49	All	gi|13786156	RNA Processing	Yes [177; 180]

Since cultured endothelial cells produce NO under basal conditions, a relatively large population of S-nitrosated proteins can be detected. In accordance with the established lack of gel-to-gel variability in 2DGE [172], we were able to identify a population of proteins that are S-nitrosated regardless of the treatment condition (Figure 3.2). In addition we were able to identify several proteins that appeared to be newly nitrosated upon exposure to NO donor (Figure 3.2).

It is of note that even large doses of NO donors (100 μM SNCEE and 200 μM S-NO Albumin) did not result in large changes in the overall S-nitrosation profile of RPAEC. However, we identified both proteins that have not been previously recognized to be S-nitrosated in general (Table 3.1) and proteins that had been identified in other studies of S-nitrosation (Table 3.2).

3.2.5.3 Detection of S-nitrosated proteins in endothelial cells from multiple species

In addition to RPAEC, we exposed human aortic endothelial cells (HAEC), mouse lung endothelial cells (MLEC), rat microvascular endothelial cells (RMEC), and sheep pulmonary artery endothelial cells (SPAEC) to NO donors and identified S-nitrosated proteins. We were able to detect S-nitrosated proteins that are involved in a variety of cellular processes (Figure 3.3).

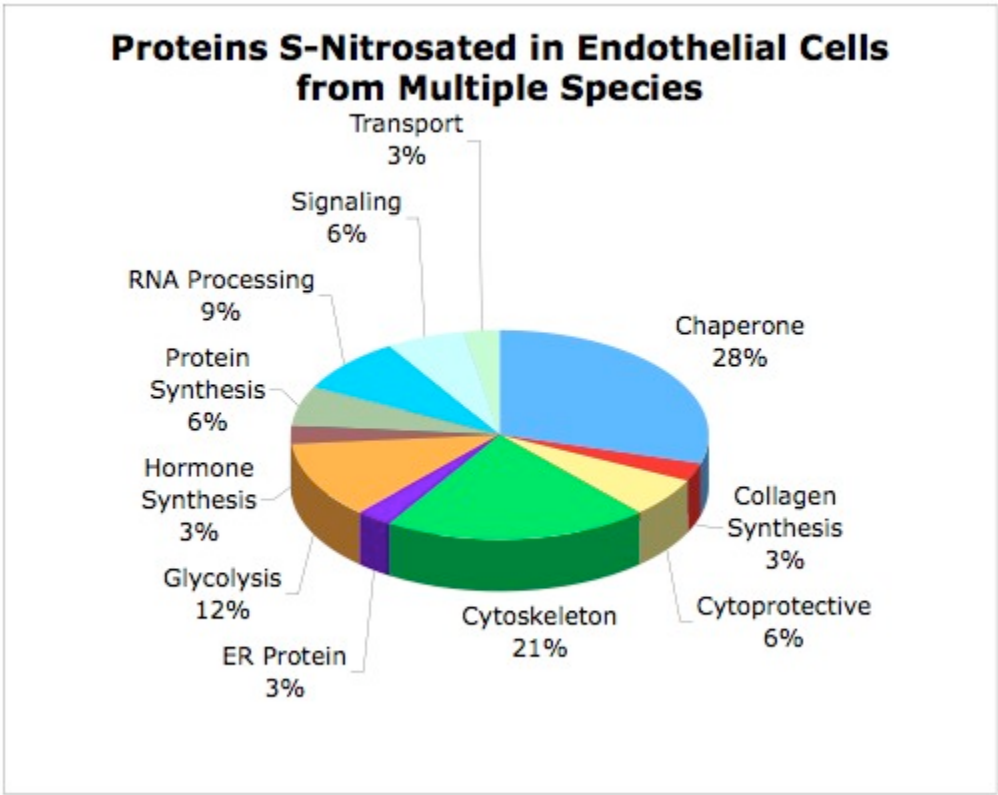

Figure 3-3 Categories of S-nitrosoproteins identified in all species of endothelial cells studied. S-nitrosoproteins identified in human, mouse, rat, and sheep endothelial cells are represented according to protein function.

It is interesting to note that two major groups of modified proteins emerged in this study, chaperone and cytoskeletal proteins. In this respect our study agrees with previous studies that

identified members of these same groups of proteins as being S-nitrosated. As in the studies in RPAEC we were able to identify both proteins that have not been previously recognized targets of S-nitrosation in endothelial cells (Table 3.3) as well as proteins that had been identified in previous studies of S-nitrosation (Table 3.4), some of which were performed using cultured endothelial cells.

Table 3-3 Proteins not previously known to be S-nitrosated as identified by MS analysis in human, mouse, rat, and sheep endothelial cells. Abbreviations: HA=human aortic endothelial cells; M=mouse lung endothelial cells; RM=rat microvascular endothelial cells; S=sheep pulmonary artery endothelial cells

Protein	**MW**	**PI**	**Species**	**Conditions**	**Accession Number**	**Function**
Endo PDI (Thioredoxin domain containing protein 5 isoform 2	44.4	5.77	HA	SNCEE	gi|42794775	Chaperone
HOP1	63.0	6.40	HA	SNCEE	gi|38181876	Chaperone
Heat shock protein 47 – non-inhibitory serpin	46.6	8.9	RM	Baseline	gi|51450	Chaperone
Heat shock protein 1	58.0	5.35	RM	All	gi|133428	Chaperone
Aldolase A	39.6	8.31	S	All	gi|202837	Glycolysis
hnRNP A2/B1/B0	37.4	9.0	S	All		RNA processing
hnRNP A3	37.2	8.5	S, M	All		RNA processing

Table 3-4 Proteins known to be targets for S-nitrosation as identified by MS analysis in human, mouse, rat, and sheep endothelial cells. Asterisks indicate proteins identified as targets of S-nitrosation in previous endothelial cell studies. 1=Result in human aortic endothelial cells 2=Result in mouse lung endothelial cells Abbreviations" HA=human aortic endothelial cells; M=mouse lung endothelial cells; RM=rat microvascular endothelial cells; S=sheep pulmonary artery endothelial cells

Protein	MW	PI	Species	Conditions	Accession Number	Function	Known S-NO Modified
B-tubulin	50.3	4.79	RM	SNO-Albumin	gi|62078507	Cytoskeleton	Yes [77; 159]
Heat shock protein 60	61/3	5.70	HA	SNCEE	gi|77702086	Chaperone	Yes [177]
Enolase 1	47.5	7.01	HA	SNCEE	gi|4503571	Glycolysis	Yes [173; 177]
Heat shock protein 70	72.4	5.07	HA, M, RM	All, SNCEE[1]	gi|38303969	Chaperone	Yes [173; 179]
Vimentin	53.7	5.06	HA, M, RM, S	All, SNCEE[1]	gi|57480	Cytoskeleton	*Yes [175]
Gamma actin	59.0	5.67	M	All, PI Shift with SNCEE[2]	gi|109492380	Cytoskeleton	*Yes [77; 159; 176]
Peroxiredoxin 1	22.3	8.27	RM	All	gi|56789700	Cytoprotective	*Yes [175; 176; 179; 181]
B-actin			RM	All	gi|4501885	Cytoskeleton	*Yes [77; 159; 175; 176; 177]
hnRNP A/B	36.3	6.49	RM	All	gi|13786156	RNA processing	Yes [177; 180]
Albumin	71.0	5.8	S	All	gi|162648	Transport/Storage	Yes [159]

In both our studies of RPAEC and other endothelial cell species, some of the targets of S-nitrosation remain the same regardless of species as evidenced by their identification in multiple species.

3.2.5.4 S-nitrosoprotein profile of sheep pulmonary artery endothelial cells exposed to an endogenous source of nitric oxide

In order to examine the effects of a more physiologically relevant source of NO we infected SPAEC with an adenovirus expressing the human cDNA for inducible nitric oxide

synthase (AdiNOS; SPAEC.iNOS). Uninfected SPAEC (SPAEC) and SPAEC.iNOS were cultured for 72 hours post-infection either in the presence or absence of the NO inhibitor L-NAME. Uninfected SPAEC were capable of producing NO under our cell culture conditions, and this NO production was enhanced upon infection with AdiNOS (SPAEC.iNOS; Figure 3.4). In both SPAEC and SPAEC.iNOS, culture in the presence of L-NAME resulted in decreased, but still detectable, NO production (Figure 3.4).

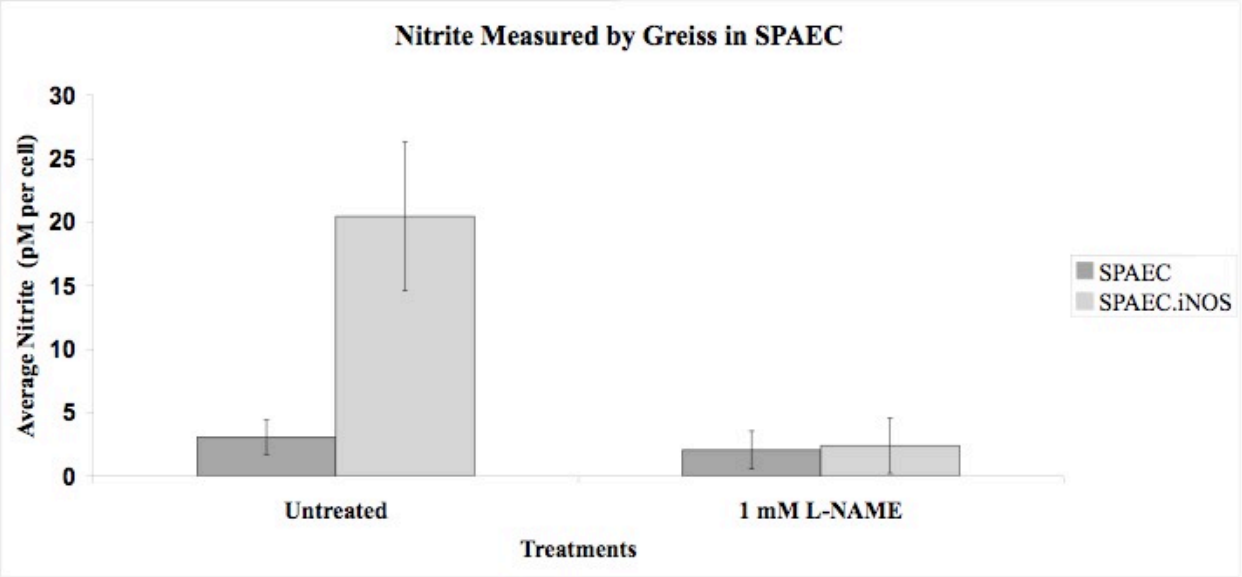

Figure 3-4 Nitric oxide production in sheep pulmonary artery endothelial cells. Nitric oxide production in uninfected sheep pulmonary artery endothelial cells (SPAEC) and sheep pulmonary artery endothelial cells infected with a 1000:1 MOI of AdiNOS (SPAEC.iNOS) was measured as nitrite in the medium using the Greiss Assay. SPAEC and SPAEC.iNOS were cultured in the presence and absence of 1 mM L-NAME, a nitric oxide synthase inhibitor.

Upon 2DGE analysis of S-nitrosated proteins from SPAEC and SPAEC.iNOS cultured with and without L-NAME we observed several phenomena. First, as in our previous studies with various types of endothelial cells, we observed a set of proteins that are S-nitrosated in

endothelial cells under basal conditions (Figure 3.5). In cells exposed to L-NAME there are no appreciable differences in the profile of proteins that are S-nitrosated (Figure 3.5).

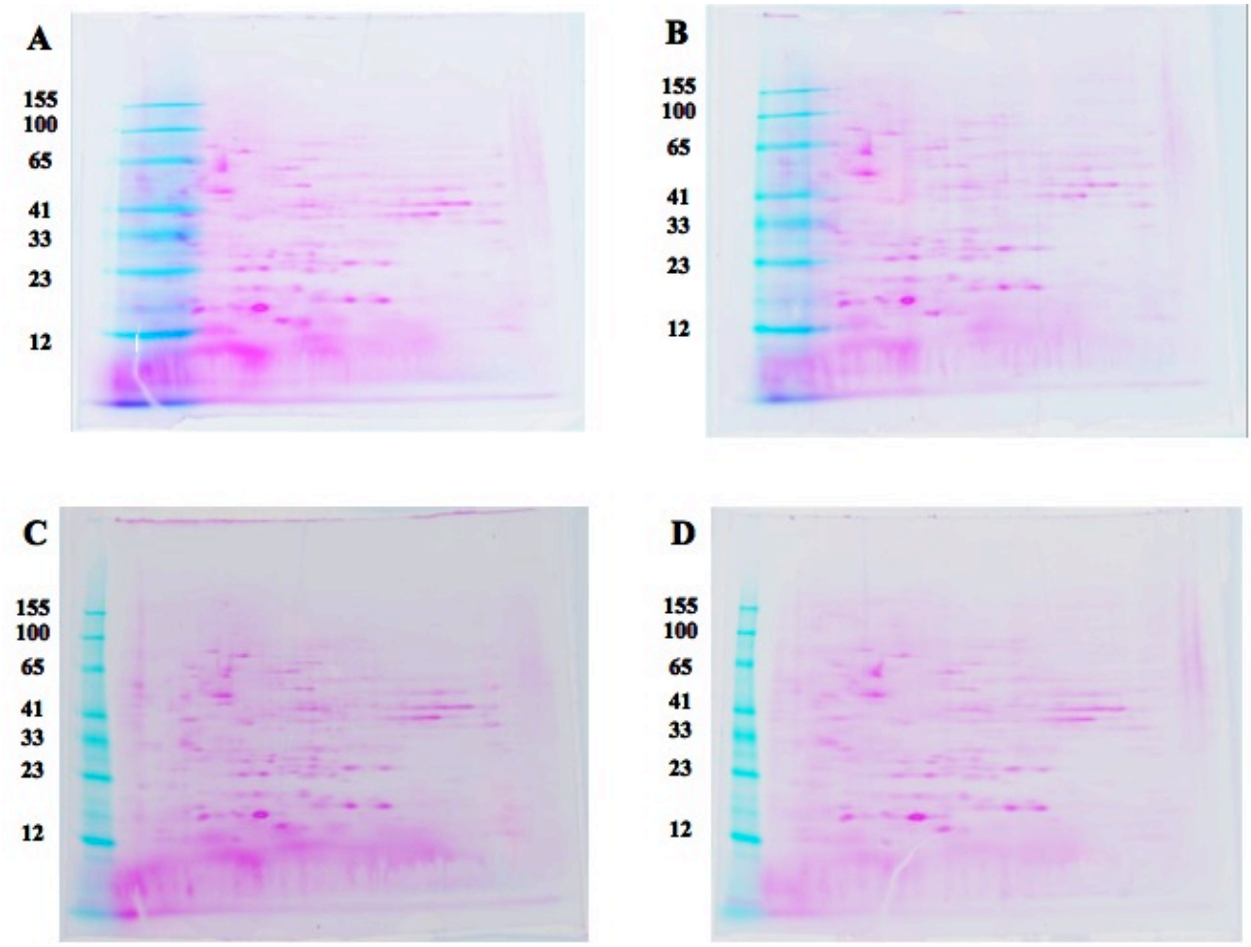

Figure 3-5 S-nitrosoprotein profile of SPAEC and SPAEC.iNOS. SPAEC were infected with a 1000:1 MOI of AdiNOS and protein was harvested 72 hours post-infection. Protein samples were used in the S-nitrosoprotein identification assay and 2DGE as described in methods. A) SPAEC at baseline conditions; B) SPAEC.iNOS; C) SPAEC exposed to 1 mM L-NAME for 72-hours; D) SPAEC.iNOS exposed to 1 mM L-NAME for 72-hours post-infection.

In addition, although nitric oxide levels were increased in SPAEC.iNOS, there were no significant changes in the spectrum of S-nitrosated proteins in these cells, even upon exposure to L-NAME (Figure 3.5). Mass spectrometry analysis of protein spots picked from this series of

gels identified both proteins that have not been previously known to be S-nitrosated in endothelial cells (Table 3.5) and proteins that have been known to be S-nitrosated in various cell types (Table 3.6).

Table 3-5 Proteins not previously known to be S-nitrosated as identified by MS analysis. Cell lysates were harvested from SPAEC and SPAEC.iNOS 72 hours post-infection and used in the S-nitrosoprotein detection assay.

Protein	MW	PI	Conditions	Accession Number	Function
Profilin	15.1	8.5	All	gil1127241	Cytoskeleton
ERp60 (PDI family	63.0	6.4	All	gil1083063	Protein Folding

Table 3-6 Proteins known to be targets for S-nitrosation as identified by MS analysis in this study. Results are for SPAEC and SPAEC.iNOS lysates harvested 72-hours post-infection.

Protein	MW	PI	Conditions	Accession Number	Function	Known S-NO Modified
Tropomyosin 4	28.6	7.14	All	gil4507651	Cytoskeleton	*Yes [176; 178]
Vimentin	53.7	5.06	All	gi|57480	Cytoskeleton	*Yes [175]
GAPDH	36.0	7.63	All	gi|56611127	Glycolysis	*Yes [77; 159; 175; 176; 177; 178; 179]
Aldehyde dehydrogenase	54.9	7.14	All	gil974168	Glycolysis	Yes [159]
Eukaryotic translation initiation factor 5A2	17.1	5.38	All	gi|76827038	Protein synthesis	Yes [92; 179]

It is interesting to note that in this set of experiments the proteins identified were consistent with those identified in our previous experiments with exogenous NO donors in a variety of species of endothelial cells (Tables 3.1-3.4).

3.2.6 Discussion

In the present study we add to the growing database of protein targets of S-nitrosation. In particular, our studies in endothelial cells, with their ability to produce endogenous nitric oxide, add weight to the growing importance of S-nitrosation as a potential post-translational signaling pathway. Using endothelial cells from a variety of species we were able to identify both potential new targets of protein S-nitrosation and known targets of S-nitrosation. Some of the proteins identified in this study have potential connections to nitric oxide.

Previous studies have identified the actin cytoskeleton as a target for S-nitrosation [182]. The authors of these studies indicated that actin could be S-nitrosated using GSNO as a NO donor and that S-nitrosated actin polymerizes less efficiently than unmodified actin. It is known that the activity of endothelial nitric oxide synthase (eNOS) is partially dependent upon the state of the cytoskeleton; particularly in shear stress-mediated NO production [39]. In endothelial cells experiencing shear stress actin microfilaments, microtubules and vimentin intermediate filaments rearrange. This disruption of endothelial cell cytoskeletal components, particularly actin, results in increased stability of eNOS mRNA [39], perhaps through binding of β-actin itself to the mRNA. Finally, there is evidence that S-nitrosated actin can function as a potent NO donor [182].

In addition to S-nitrosated actin, we were able to identify other elements of the cytoskeleton as potential targets for S-nitrosation. In particular we identified tropomyosin, a protein that has been demonstrated to be one of the most abundant components of the cytoskeleton in bovine pulmonary artery endothelial cells [183]. Other studies have demonstrated that cell-surface tropomyosin may play a role in preventing angiogenesis through its interactions with other extracellular proteins [184].

We were able to identify several isoforms of protein disulfide isomerase (PDI) in our studies. Previous evidence from our laboratory and others demonstrates a link between PDI and NO [83; 87; 185]. PDI isoforms are members of the thioredoxin superfamily, and while they can be found at the cell surface, they are mainly present in the lumen of the endoplasmic reticulum [186]. There are several studies that indicate that PDI is involved in transport of S-nitrosothiols from the extracellular to the intracellular space [83; 87; 185]. In addition, in this study we were able to identify an endothelial cell-specific PDI isoform, EndoPDI, as a target for S-nitrosation [187]. Finally, it has been demonstrated that PDI is a subunit of prolyl-hydroxylase [188], another protein that we identified as a potential target of S-nitrosation in our study.

We were also able to identify several proteins that may be involved in linking NO to apoptosis in endothelial cells. It is known that NO has both pro and anti-apototic effects in cells depending upon the circumstances [154]. In our studies we were able to identify two proteins that other groups have determined to be targets of S-nitrosation in other cell types and that are known to be involved in apoptotic pathways involving nitric oxide.

The first protein that has been identified as a target of S-nitrosation in this study and others is eukaryotic translation initiation factor 5A2 (eIF5A2) [92; 179]. eIF5A2 is the only protein known to use the amino acid hypusine, a spermine-modified lysine, to stimulate the formation of the first peptide bond during the translation process [189; 190]. It has been suggested that rather than participating in global translation of proteins, eIF5A2 is specifically involved in translation of proteins that are involved in both apoptosis and proliferation in endothelial cells [189].

In addition to eIF5A2, GAPDH has been identified as a target of S-nitrosation in our study and several others [77; 159; 175; 176; 177; 178; 179]. There have been many studies of

the relationship between GAPDH and caspase independent cell death [191]. Of particular interest in this pathway is the relationship between NO, GAPDH and the protein Siah1. In a study using LPS activated macrophages it was shown that NO production leads to S-nitrosation of GAPDH [192]. This NO-based modification of GAPDH allows it to bind to Siah1, an E3 ubiquitin ligase, and using Siah1's nuclear translocation signal the complex migrates to the nucleus. Under normal conditions Siah1 is rapidly degraded by the ubiquitin proteasome system, but it is stabilized by complexing with GAPDH, resulting in increased nuclear protein degradation and ultimately cell death via apoptosis [193; 194].

In addition to identification of novel targets of S-nitrosation, this study allowed us to make comparisons of the utility of our modification of the biotin-switch assay in identifying targets of S-nitrosation in endothelial cells using both exogenous and endogenous sources of NO. In the first set of studies we used relatively high, non-physiological doses of two well characterized NO donors, S-nitrosoalbumin and L-SNCEE [42; 61; 83; 127; 170; 171; 195]. In this case, where endothelial cells capable of producing low, steady-state concentrations of NO in culture (Figure 3.4) were exposed to large doses of NO from an external source, we were able to observe changes in the S-nitrosation state of several cellular proteins. In contrast, when endothelial cells were exposed to a more physiologically relevant source of NO by infection with AdiNOS, while overall NO production was increased slightly (Figure 3.4), any changes in the spectrum of S-nitrosated proteins were masked by the steady state production of NO by endothelial cells in culture. In addition, we were unable to fully inhibit NO production in culture. These experiments lead us to conclude that the modified biotin-switch method is an effective tool to monitor large changes in the S-nitrosation state of proteins, but more subtle changes may be lost in the "background" created by steady state NO production in endothelial

cells. Despite the limitations of the assay we were able to use our modified assay to allow more efficient proteomic analysis of S-nitrosated proteins from complex mixtures and to identify novel targets of S-nitrosation in endothelial cells.

This technique also allowed us to extend our studies of the potential for S-nitrosation of metallothionein. Previous studies in our laboratory using a fluorescence resonance energy transfer-based metallothionein reporter have shown that exposure of this reporter to endogenous and exogenous sources of NO results in zinc release from metallothionein and consequent unfolding of the reporter construct [60; 61; 62]. We attributed these NO-mediated changes to S-nitrosation of the protein thiol groups present in MT. In this study we used our S-nitrosation detection assay in combination with immunoblotting to determine that MT is in fact S-nitrosated in endothelial cells (Figure 3.1). These experiments add weight to the conclusions from previous studies and indicate that S-nitrosation of thiol residues in MT plays an important role in NO-mediated zinc signaling in endothelial cells.

This study has demonstrated that a modified version of the biotin-switch method may be used effectively to screen for NO-mediated changes in the S-nitrosation state of the proteome of endothelial cells. However, this method, like any method based upon the biotin-switch method has several caveats. There are a variety of forms that NO-mediated thiol modification may take including glutathionylation and disulfide formation [106]. The biotin-switch method is not meant to distinguish between these modifications and a S-nitrosothiol. In addition, its sensitivity has been questioned. It has been reported that in order to be detected by this method, S-nitrosothiols must be present in at least nanomole per milligram of protein levels [106; 118]. Given the potential problems with the biotin-switch method and its derivatives the S-nitrosation state of any proteins identified in a proteomic screen must be verified using a secondary method

of detection, with mass spectrometry to identify the modification of a specific thiol group being the gold standard [77; 94; 196]. Regardless of the limitations of the assay, general consensus within the field states that methods derived from the biotin-switch can be valuable screening tools if used carefully and if the results are ultimately verified using another method [106; 117]. As we have demonstrated in this study, a fluorescent modification of the biotin-switch assay has allowed us to identify S-nitrosated proteins in endothelial cells using a proteomic approach. This tool will be a valuable addition to the arsenal of S-nitrosoprotein detection techniques and ultimately may be used to help us further dissect NO-mediated signaling pathways in endothelial cells.

4.0 CHAPTER 4: MEASUREMENT OF PROTEIN S-NITROSOTHIOLS IN THE VASCULATURE

In preparation

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4.1 INTRODUCTION

In this article we discuss and compare existing methods of S-nitrosothiol detection. We examine traditional S-nitrosothiol detection assays such as flash-photolysis chemiluminescence, chemical reduction assays, and fluorometric measurement methods. However, the main focus of the article is on newly emerging methods to examine to S-nitrosoproteome. We discuss the biotin switch method for S-nitrosothiol detection and its potential limitations. We also present our

experience detecting S-nitrosoproteins in endothelial cells and the additional complications that working with this cell type adds to S-nitrosothiol detection.

4.2 MANUSCRIPT AS PREPARED FOR SUBMISSION

4.2.1 Abstract

This review discusses the measurement of S-nitrosothiols in proteins with an emphasis on a recently developed technique that facilitates analysis of the S-nitrosoproteome in tissues and cell lines. Protein S-nitrosation has been proposed as a newly emerging, post-translational protein modification-based signaling pathway for nitric oxide. As researchers continue to evaluate the relevance of protein S-nitrosation as a signaling pathway, an understanding of all of the tools available for S-nitrosothiol analysis will become increasingly valuable.

4.2.2 Keywords

Nitrosation

Biotin Switch

S-nitrosthiols

Nitric Oxide

Thiols

4.2.3 Introduction

Nitric oxide (NO) is formed by nitric oxide synthases (NOSs) as a gaseous free radical. Expression of NOS, and therefore production of NO, can be either constitutive or inducible [157]. As such, NO's function varies according to cell type. In the vasculature NO is produced by endothelial NOS and functions as endothelium derived relaxation factor. This vasodilatory function of NO is a result of the classical NO signaling pathway in which NO interacts with the heme in guanylate cyclase ultimately leading to increased production of cyclic guanosine monophosphate (cGMP). The increased levels of cGMP activate cGMP-dependent protein kinases leading to activation of downstream signaling factors [197]. For many years NO was thought to exert its action solely through this role as a diffusible second messenger that affects cellular processes strictly through its effect on cGMP levels. Recently a new NO signaling paradigm has emerged. The S-nitrosation of protein cysteine-thiols is thought to be a new post-translational protein modification with potential signaling consequences analogous to protein modification via phosphorylation [93].

4.2.4 Protein S-nitrosation as a nitric oxide-mediated signaling pathway

There is mounting evidence that NO can react with proteins to post-translationally modify thiol groups. These modifications are not dependent upon NO itself, but rely on the formation of nitric oxide derivatives in biological systems to exert this nitrosating effect [176]. It is also prudent to mention that S-nitrosothiols are not the only NO-based modification that may be formed in biological systems. Both the addition of an NO group to tyrosine residues (tyrosine

nitration) and methionine oxidation are examples of cGMP-independent NO modifications of proteins [34]. In addition to S-nitrosation, cysteine residues may also undergo oxidation to sulfenic, sulfinic, and sulfonic acid, or addition of a glutathione moiety (S-glutathionylation) [34]. However, in this review we will focus on S-nitrosation of protein thiols.

There are several theories in the literature regarding the function of protein S-nitrosothiols. Many are willing to accept the possibility that protein S-nitrosation represents a unique signaling pathway in biological systems [76; 77; 93; 159], however there are other arguments proposing that protein S-nitrosothiols serve as a NO transport mechanism via transnitrosation or as a NO reservoir in cells [42; 198]. Finally there are groups who propose that protein S-nitrosation occurs most prevalently when cells undergo oxidative stress and glutathione levels become depleted [199; 200; 201]. In biological systems the reaction between NO and thiols is unfavorable, with metal centers and free radicals being kinetically preferable targets [202; 203]. Despite the kinetic barriers, S-nitrosothiols can be detected in vivo in both normal and pathological conditions [33; 42; 104; 204].

4.2.5 Formation of S-nitrosothiols in vivo

There are four main mechanisms by which S-nitrosothiols may be formed in vivo. The first involves the reaction of nitrous acid (HONO) with thiols. This is in fact the major mechanism of S-nitrosothiol formation in vitro [117], but due to the very low pH levels required, it is unlikely to contribute significantly to protein S-nitrosation in vivo. It may be involved in formation of S-nitrosothiols in the gastrointestinal tract [117].

The second method of S-nitrosothiol formation involves direct addition of a nitrosonium cation (NO^+) or its equivalent to a thiol group. There is evidence that peroxidase complexes I and II can be reduced by NO and form NO^+ [205; 206], however NO^+ is very unstable in aqueous solution at neutral pH so the target thiol must be in extremely close proximity to the site of NO^+ production [117].

The third mechanism of S-nitrosothiol formation is a result of direct nitrosation by N_2O_3. This reaction is relatively fast [117], however formation of N_2O_3 requires NO to be oxidized to NO_2 and then to combine with a second NO molecule and this reaction proceeds rather slowly at biological concentrations of NO. Some have suggested that NO and O_2 may be concentrated in hydrophobic membrane and protein pockets [95; 96] and that this might accelerate the formation of N_2O_3 thereby increasing the relevance of this pathway in protein S-nitrosation in biological systems.

The fourth potential mechanism for formation of protein S-nitrosothiols involves direct combination of a NO radical with a thiyl radical in a diffusion rate limited radical-radical combination reaction [207; 208]. Since it has been demonstrated that glutathione is able to repair other free radicals it is possible that thiyl radicals may be formed as a result of oxidative free radical exposure [117].

Finally, the most important reaction of S-nitrosation when discussing its potential as a signaling pathway is the reversible transfer of the nitroso group from an S-nitrosothiol to a target thiol. This reaction is known as transnitrosation. In most cases the rate of transnitrosation in biological systems is determined by the pKa of the attacking thiol group, as pKa is one determination of the nucleophilicity of the thiol group [209; 210]. Low molecular weight (LMW) thiols are able to modify protein thiols and to convert the protein nitrosothiol back to the

original thiol. The distribution of the S-nitroso groups within a cell will depend upon the rates of all possible S-nitrosation reactions within in the cell, which in turn is highly dependent upon the pKa of the thiols involved; the lower the thiol pKa, the more likely it is to become a target for transnitrosation [209].

Rather than being a random process as the discussion above might suggest, protein S-nitrosation is thought to be restricted to specific thiols on the target protein by several potential mechanisms. The first mechanism stems from the theory that acidic and/or basic amino acids surrounding the modified thiol can alter its pKa, rendering it more nucleophilic and therefore more susceptible to S-nitrosation [93], as was initially described in the protein hemoglobin [211]. In other proteins where an acid/base motif has been identified it is composed of flanking acidic (Asp, Glu) and basic (Arg, His, Lys) residues [81]. The proximity of the acidic and basic residues in the tertiary and/or quaternary structure of the protein is key to the success of the so-called acid-base motif, as evidenced by recent studies that attempted to predict sites of S-nitrosylation based solely on the linear amino acid sequence of a particular protein [94]. A second mechanism thought to lead to the tight control of S-nitrosation is the subcellular compartmentalization of sources of NO and target proteins based upon interactions with nitric oxide synthases (NOSs) or NOS associated proteins [81]. Finally, the presence of hydrophobic pockets in a protein may also lend specificity to S-nitrosation [91]. This idea is supported by the fact that NO and O_2 tend to preferentially concentrate in hydrophobic areas, as evidenced by the increase in reaction rate between the NO/O_2 pair in the hydrophobic environment of the plasma membrane [95]. Additional studies have shown that hydrophobic pockets within proteins lead to increased S-nitrosation of the cysteine residues found there [96].

4.2.6 Dysregulation of protein S-nitrosation and disease

There is evidence that an imbalance in protein S-nitrosation may play a role in human diseases [30]. Elevated levels of s-nitrosation may contribute to diseases such as arthritis, sepsis, preeclampsia, and hypercholesterolemia, while decreased levels of protein S-nitrosation have been implicated in diseases such as asthma, cystic fibrosis, and pulmonary hypertension. In each case, it seems as if an alteration in protein post-translational modification (S-nitrosation) may result in the malfunctioning of cellular signaling pathways [30].

4.2.7 Common analytical methods for S-nitrosothiol measurement

There are a number of challenges to be faced when attempting to measure S-nitrosothiols in biological systems mostly due to their very low levels in vivo. Various reports have been made regarding the concentration of S-nitrosothiols in biological systems. The values range between 1.5 – 2 mM in endothelial cells [80] and rat kidney [80] to 10 – 100 nM in various tissues as measured by Bryan et al. [212]. This four- to-five order of magnitude gap in various measurements in the literature can be seen as an illustration of the difficulty of accurately measuring S-nitrosothiols in biological systems. Since S-nitrosothiols are present at such low levels in vivo the presence of nitrite, iron-nitrosyl and other contaminants can serve as significant artifacts in any measurement system [105]. An additional complicating factor is introduced when one considers the lability of the S-NO bond. Protein S-NO bonds are extremely sensitive to changes in tertiary and quaternary structure of the protein, as well as preparation conditions

after extraction [107]. Care must be taken not to extensively modify the level of protein S-nitrosation through the assay conditions used to quantify protein S-nitrosothiols [213; 214].

4.2.8 S-nitrosothiol detection techniques

There are a number of detection techniques that are commonly used to measure S-nitrosothiols (Table 4.1). We will discuss each of the techniques briefly and their application to measurement of protein S-nitrosation in complex mixtures.

Table 4-1 Common S-nitrosothiol detection techniques. Modified from [108].

Method	Mechanism	Detection Limit	Selectivity	Pros	Cons
Photolysis-Chemiluminescence [78; 114; 198; 215]	Photolysis induced cleavage of S-NO bond	pM range	Nitrite and iron-nitrosyls are also detected	Sensitive	Equipment is very expensive Detection of false positives possible Can not detect S-nitrosothiols in a specific protein of interest in a complex sample
Reductive chemiluminescence [110; 114; 216; 217; 218]	Copper and cysteine selectively reduce S-NO bond	pM range	Selective for S-nitrosothiols	Both specific and sensitive	Nitrite can interfere with assay at low pH Presence of heme can obscure detection of S-nitrosothiols Can not detect S-nitrosothiols in a specific protein of interest in a complex sample

Table 4-1 continued

Method	Mechanism	Detection Limit	Selectivity	Pros	Cons
Nitrite quantification using KI chemiluminescence [110; 114; 219]	Thiol bound NO converted to NO^+ and detected as nitrite after reduction by KI in acidic solution	pM range	Not selective – indirect measurement	Simple assay	Not very sensitive – should not be used as stand alone assay Can not detect S-nitrosothiol on a particular protein of interest in a complex sample
Saville assay [209; 220]	Thiol bound to NO must be converted to NO^+ and is then detected using Greiss reagent	Approx 500 nM	Selective for S-nitrosothiols	Simple and inexpensive	Not a sensitive measurement Can not detect S-nitrosothiols in a particular protein of interest in a complex sample
DAF-based fluorescence [217; 221; 222]	NO^+ based conversion of DAF to fluorescent DAF-2T	nM range Gel – based methods μM range	Can be sensitive depending upon conditions	Can be used in high-throughput systems	S-NO must be quantified in the presence and absence of $HgCl_2$ Can not detect S-nitrosothiols on a particular protein of interest in a complex mixture
Mass spectrometry [77; 94; 196; 223; 224; 225; 226; 227]	Monitor appropriate m/z	Dependent upon instrument Usually in 200 nM range in solutions	Highly selective	Highly selective If paired with LC/MS can detect S-nitrosothiols in a particular protein of interest in a complex mixture	Must design LC phase to stabilize S-NO bonds and avoid creating new S-NO bonds Must use internal standards labeled with ^{15}N
Biotin switch method [77; 106; 116; 117]	Use biochemical substitution to biotinylate S-nitrosated cysteine moieties in proteins		Selective for S-nitrosothiols	Selective if proper reaction conditions and controls are used	Can give spurious results if conditions are not carefully controlled Must be verified using a second detection method

The first technique that is often used to measure S-nitrosothiols is photolysis-chemiluminescence [78; 198]. In this technique the S-N bond of nitrosothiols is homolytically cleaved using high intensity UV light to liberate both a NO and a thiyl free radical which is detected by reaction with ozone in the gas phase by a nitric oxide analyzer [107]. This process is most often used to detect the total amount of S-nitrosothiols in a sample or a purified protein and thus is not in itself capable of determining the S-nitrosation status of an individual protein in a complex mixture such as the proteome. In addition, it has been demonstrated that photolysis is not necessarily specific for cleavage of the S-NO bond. It can also release nitroso groups from nitrosamine and iron-nitrosyl species, and reduce nitrite to NO in the presence of thiols, which can lead to an overestimation of the S-nitrosothiol content of a sample [108; 109].

A second set of S-nitrosothiol measurement assays depend upon reduction of the S-NO bond either with CuCl and cysteine [49; 110; 216; 217] or potassium iodide (KI) [110; 114; 205]. In the first case NO is measured via reaction with ozone in a nitric oxide analyzer. In the second case the thiol bound NO is detected as nitrite after reduction with and acidic solution of KI. Both of these methods have serious limitations. In the case of CuCl chemiluminescence the presence of nitrite at low pH can be observed as part of the S-nitrosothiol signal. In addition the presence of heme in the assay can mask the S-nitrosothiol signal [107]. In the case of KI reduction, the method is simple, but cannot be used as a stand-alone assay [107; 114]. Finally, neither assay can be used in analysis of complex mixtures such as the proteome.

The third method, the Saville-Greiss method, uses conversion of the S-nitrosothiol bond to nitrite, which is detected colorimetrically using the Greiss reagent [114; 209; 220]. This method is very insensitive, with its limit of detection around the 500 nM range. It is also not applicable to analysis of the proteome.

The fourth method is a fluorescence-based assay dependant upon the conversion of non-fluorescent 4,5-diaminofluorescein (DAF) to fluorescent DAF-2T upon exposure to NO^+ [217; 221; 222]. However, fluorescent methods suffer from lack of specificity and reproducibility [104]. In addition, this method is commonly used to identify total S-nitrosothiols in a sample or purified protein, making it unsuitable for analysis of individual S-nitrosothiols in a complex sample.

The final commonly used method to analyze S-nitrosothiols in a sample is mass spectrometry [77; 92; 94; 196; 223; 224; 225; 227; 228]. Mass spectrometry based methods are extremely sensitive, and if samples are processed carefully, very accurate [107]. In addition to the sensitivity of these methods, when used in combination with high-pressure liquid chromatography they can be used to examine the S-nitrosation status of individual proteins within a complex mixture such as the proteome [92; 94]. However, not all researchers have access to the mass spectroscopic equipment necessary to perform proteomic analyses strictly based upon mass spectroscopic analysis of tissue preparations or cell lysates. In this case another method for analysis of individual S-nitrosoproteins in a complex mixture, known as the Biotin Switch Assay [77; 116] offers researchers another alternative.

4.2.9 The biotin switch assay and modifications

One of the current methods for identifying s-nitrosated proteins in a complex cellular milieu is the biotin-switch assay, developed by Jaffrey et al[77; 116]. The method results in the specific labeling of s-nitrosated proteins with a biotin moiety or a fluorescent tag on S-nitrosated cysteines [116] (Figure 4.1, noted in black font).

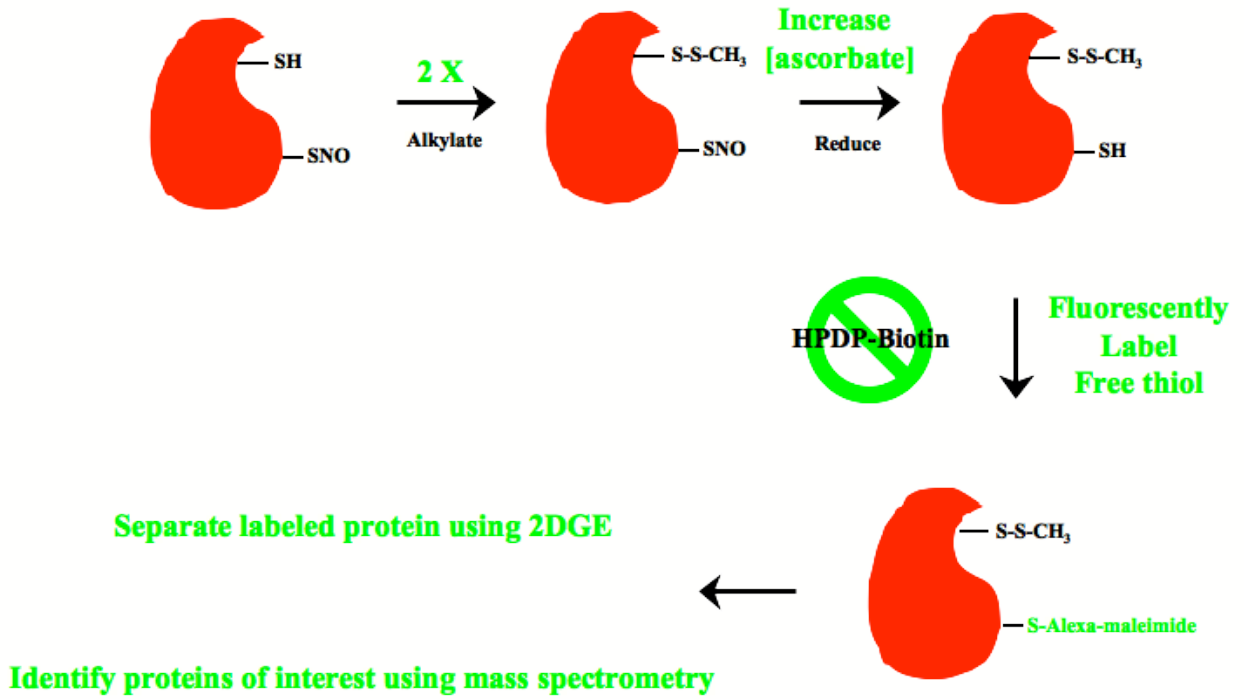

Figure 4-1 The Biotin Switch Method for detection of protein S-nitrosothiols. The steps from the original assay are noted in black. Modifications to the original assay are noted in green.

This process involves three sequential steps. First, free thiols are blocked by incubating protein mixtures with methyl methane thiosulfonate (MMTS), a thiol-specific methylating agent. Sodium dodecyl sulfate is included to denature proteins and allow MMTS to access internal thiol residues. The remaining protein nitrosothiols are reduced to thiols using ascorbate. Finally, the resultant thiols are reacted with a biotin moiety (*N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide; biotin-HPDP) resulting in selective labeling of s-nitrosothiols[116]. Using Western blotting, biotin-labeled proteins can be revealed using an anti-biotin antibody [77] or streptavidin-peroxidase[176]. When combined with proteomic approaches, this assay can be used to explore the extent of s-nitrosation in tissues or cultured cells.

As is the case with other s-nitrosothiol detection methods, the biotin-switch assay has several potential pitfalls. The sensitivity of the assay is dependent upon both the ability to effectively block free thiols to render them inert, and the effectiveness of ascorbate as a reducing agent. Both MMTS[77] and *N*-ethylmaleimide (NEM) [229] have been used to effectively block samples, reducing the background upon subsequent Western blotting to very low levels. In addition, studies have shown that ascorbate does not reduce all S-nitrosothiols with equal affinity [230; 231; 232]. In fact, a recent study by Zhang et al.[118] demonstrated that the sensitivity of the biotin-switch assay was dependent upon both ascorbate concentration and incubation time. It has been suggested that reactivity of S-nitrosothiols toward ascorbate may be influenced by amino acid sequence, thiol pK_a, and steric hindrance [118]. To date these issues have not been thoroughly studied, thus it appears that modifications to the original biotin-switch protocol may increase sensitivity [118].

To that end our laboratory and others have made several modifications to the original method (Figure 4.1, noted in green font) [92; 94; 117; 118; 175]. Increasing the number of MMTS blocking steps in the original protocol can decrease the detection of background signals from incomplete blocking. In addition, increasing the concentration of ascorbate and the incubation time used in the reduction step can lead to increased sensitivity in the assay [117; 118]. Finally, the use of thiol-specific fluorescent labels can increase the efficiency of proteomic analyses using the assay (Stitt et al., unpublished data) [117].

4.2.10 Potential limitations of the biotin switch assay

There are certain caveats that must be considered whether one uses the original Biotin Switch Assay or a modification of the original. It is important to recognize that the Biotin

Switch Assay can serve as a valuable first screening tool to examine the S-nitrosoproteome of a particular tissue or cell type. However there are questions related to the specificity of the assay. For example, some groups have discovered that disulfide bonds present in proteins may be reduced with ascorbate during the protein processing necessary for the assay and may therefore be identified as potential S-nitrosated proteins [233]. It is also possible that the assay may detect sulfenic and sulfinic acid thiol modifications as well as cystinylated and glutathionylated proteins [106]. Therefore, any experiments performed using this assay must be carefully considered, and any potentially nitrosated proteins that are identified using the assay must be confirmed to be S-nitrosated using another common method of S-nitrosothiol measurement [106; 117; 234].

4.2.11 The biotin switch assay in endothelial cells

In addition, when performing studies in vascular cells, particularly endothelial cells, there are other considerations that are necessary. Cultured primary endothelial cells produce NO via endothelial nitric oxide synthase [23]. This leads to the formation of a group of S-nitrosated proteins that are present as a baseline in every analysis. This baseline level of S-nitrosated proteins can mask subtle changes in the S-nitrosoproteome such as those that might be hypothesized to occur upon exposure to increased NO production in inflammation for example (Stitt et al., unpublished data). More substantial changes in the S-nitrosoproteome, such as those resulting from exposure to an exogenous NO donor, can be detected with careful use of this technique. The Biotin Switch Assay shows promise, despite its limitations, as a valuable tool to analyze the potential effects of S-nitrosoprotein-based signaling pathways in the vasculature.

5.0 CHAPTER 5: DISCUSSION

5.1 NITRIC-OXIDE MEDIATED ZINC HOMEOSTASIS IN ENDOTHELIAL CELLS: A ROLE FOR METALLOTHIONEIN AND METAL RESPONSIVE TRANSCRIPTION FACTOR-1

It is known that S-nitrosation of cysteine residues capable of forming zinc complexes is an important component of NO-mediated signaling, and that MT is capable of forming a link between NO and cellular zinc homeostasis [60; 61; 62; 75]. In this study we have demonstrated that MTF-1 can serve as a component of the MT-zinc signaling pathway by affecting gene expression (Chapter 2). There is evidence in the literature that metals and oxidants can activate MTF-1 by redistributing zinc within the cell, and thereby activating expression of MT, one of the best-known gene targets for MTF-1 [67; 72; 74; 138; 143]. We have demonstrated that NO increases labile zinc levels in pulmonary endothelial cells [62], and that this causes MTF-1 to translocate to the nucleus of cells (Chapter 2, Figures 2.1-2.3). We used cells derived from MT-null mice to demonstrate that there was no NO-mediated zinc release or MTF-1 translocation, which indicates that MT plays a central role in this process.

It has been shown that NOS-derived NO and NO donors are both able to increase MT mRNA expression in bovine aortic endothelial cells [75]. We extended these finding by

demonstrating that exposing sheep pulmonary artery endothelial cells to NO increases the expression of MT protein and causes nuclear translocation of MTF-1.

We previously reported that S-nitrosation of the cysteine groups in metallothionein is a critical component of cellular redox state that links NO to Zn homeostasis in pulmonary endothelial cells [60; 61; 62]. In this study we have demonstrated that NO-induced changes in labile zinc activate the zinc-sensitive transcription factor MTF-1. It is known that NO may either contribute to or prevent apoptosis [154; 155]. We speculate that the NO-induced activation of MTF-1, which leads to increased expression of cytoprotective genes, may contribute to the anti-apoptotic effects of NO.

5.2 EXAMINATION OF THE S-NITROSOPROTEOME OF PULMONARY ENDOTHELIAL CELLS

We extended our studies of the effect of NO and post-translational S-nitrosation of protein thiol residues to the examination of the S-nitrosoproteome of pulmonary endothelial cells. We first developed a fluorescent modification of the biotin switch assay [77; 116] that allowed us to directly detect S-nitrosated proteins in a two-dimensional gel format and use peptide mass fingerprinting to identify potential proteins of interest (Chapter 3). We first used this technique to examine the S-nitrosation status of MT in sheep pulmonary artery endothelial cells exposed to NO donor (Chapter 3, Figure 3.1). We were able to use a combination of immunoblotting and two-dimensional gel electrophoresis to demonstrate that MT is in fact nitrosated in pulmonary endothelium exposed to NO donor. This reinforces our previous studies where we determined

that NO-based modification of the zinc-thiolate clusters in MT are a critical link between NO and zinc homeostasis in pulmonary endothelial cells [60; 61; 62].

We then extended our studies of protein S-nitrosation to five different endothelial cell types from four different species (human pulmonary artery endothelial cells, mouse lung endothelial cells, rat pulmonary artery endothelial cells, rat microvascular endothelial cells and sheep pulmonary artery endothelial cells) and used our fluorescent modification of the biotin switch technique to identify potential proteins of interest. The first observation of note is that the spectrum of proteins identified from endothelial cells of different species exposed to two different NO donors, L-SNCEE and S-nitrosoalbumin, is relatively consistent (Chapter 3, Tables 3.1-3.4). Since endothelial cells in culture are capable of producing NO (Chapter 3, Figure 3.4) this is not especially surprising. In fact when the results from all species of endothelial cells examined in this study are combined, several groups of proteins appear as particularly prominent targets of S-nitrosation including cytoskeletal, chaperone, and glycolytic proteins (Chapter 3, Figure 3.3).

We also examined the profile of S-nitrosated proteins in sheep pulmonary artery cells where NO production was modified by infection with an adenoviral vector expressing the cDNA for human iNOS (AdiNOS). Because endothelial cells in culture produce NO (Chapter 3, Figure 3.4) there was a significant level of protein S-nitrosation in uninfected cells (SPAEC; Chapter 3, Figure 3.5). Increased NO production via infection with AdiNOS (Chapter 3, Figure 3.4) did not result in any appreciable changes in the spectrum of S-nitrosated proteins (Chapter 3, Figure 3.5). In addition, both uninfected and infected cells treated with the NOS inhibitor L-NAME showed a similar profile of S-nitrosated proteins when compared to uninfected and infected controls (Chapter 3, Figure 3.5). This is most likely due to the fact that even in the presence of

L-NAME sheep pulmonary artery endothelial cells are capable of producing NO (Chapter 3, Figure 3.4).

It is our experience that, in endothelial cells, a fluorescent modification of the biotin switch method can be used to discern large changes in protein S-nitrosation (such as those changes caused by exposure to NO donors). However, it appears that baseline NO production in these cells can obscure smaller changes in protein S-nitrosation (such as those changes caused by increased NO production via expression of iNOS).

Regardless of the limitations encountered in our work with endothelial cells, we were able to identify new potential targets of protein S-nitrosation in this cell type. It will be necessary to verify that the potential targets that we have identified are indeed S-nitrosated using another method for measurement of protein S-nitrosothiols. However, we have developed a useful screening technique to determine levels of protein S-nitrosation in a variety of endothelial cells types. It is our hope that this work will contribute a new perspective to the function of S-nitrosothiol modifications as a signaling pathway and ultimately lead to increased understanding of how dysregulation of this pathway may contribute to diseases such as asthma, pulmonary hypertension and sepsis.

5.3 FUTURE DIRECTIONS: DISSECTING S-NITROSATION AS A POTENTIAL SIGNALING PATHWAY

There is a great deal of focus on the potential for S-nitrosation to serve as a post-translational signaling pathway somewhat analogous to protein phosphorylation. However, most studies thus far have been screening experiments meant to determine the extent of the S-nitrosoproteome in

various cell types and conditions. There is a need for more focused efforts in order to determine whether S-nitrosation can indeed function as a signaling pathway as opposed to serving as a biomarker for oxidative and nitrosative stress.

There are some studies demonstrating that S-nitrosation is capable of playing a role in specific signaling pathways. For example, the studies of the GAPDH/Siah1 interaction demonstrate a clear role for modification of a particular thiol group leading to downstream effects including apoptosis [191; 192; 193; 194]. In these studies it has been demonstrated that S-nitrosation of a particular thiol in GAPDH results in formation of a complex with the ubiquitin ligase Siah1. The complex uses the nuclear localization signal located in Siah1 to translocate to the nucleus, where protein degradation is increased, ultimately leading to caspase independent cell death via apoptosis. In this case there is a specific nitrosative signal that leads to clear downstream effects. The question that remains is one of dissecting the potential signaling interactions of proteins identified by screening methods such as the Biotin Switch Method and its modifications.

There are several possible methods available to researchers wishing to examine the role of potential protein targets of S-nitrosation in more detail. The first step in these endeavors is to verify the S-nitrosation status of the proteins identified in a screen. Traditional methods of S-nitrosothiol detection, such as flash-photolysis chemiluminescence or mass spectroscopy should be used to verify the addition of an NO moiety to a protein. These approaches are challenging since most require purification of the protein before a true measure of its S-nitrosation status can be made. In this case, mass spectrometry may prove to be the most useful verification method. S-nitrosothiols in proteins from total cellular extracts can be substituted using a modification of

the Biotin Switch Method, separated by liquid chromatography, and the substituted peptide can be identified by mass spectroscopy [92; 94].

Regardless of the method used to confirm the presence of a nitric oxide-mediated protein modification, the next step in a careful analysis would be to determine protein interaction partners that may be dependent upon the S-nitrosothiol modification. There are several approaches that may be used to accomplish this. It is possible to use a traditional immunoprecipiation method using an antibody against the NO-modified protein to pull down potential protein partners. In addition, a method based upon a yeast two-hybrid screening system has been adopted to identify protein interactions that are dependent upon NO-mediated modifications [235]. The method uses a yeast strain in which the yeast flavohemoglobin has been deleted in order to prevent it from scavenging NO. The method is analogous to a traditional yeast two-hybrid system in which the S-nitrosothiol-modified protein is used as bait and screened against a "prey" library with one extra step. During the screening process the bait and prey proteins are exposed to a NO donor in order to identify nitric oxide-mediated protein interactions [235]. The authors were able to use this technique to identify NO-dependent interactions between procaspase 3, a known target of S-nitrosation, and the proteins acid sphingomyelinase and nitric oxide synthase [235]. This method is a promising tool that could be used to identify protein partners for other proteins identified in screening of the S-nitrosoproteome.

However, the ultimate confirmation of a role for S-nitrosation of a particular protein thiol in a NO-based signaling pathway involves site directed mutagenesis of the modified cysteine thiol. If mutation of the proposed target cysteine abrogates S-nitrosation of the protein and

interaction with its potential protein partners then one can be confident that the cysteine residue in question plays an important role in a potential nitric oxide-mediated signaling pathway.

We are still in the beginning stages of understanding the role that nitric oxide may play in cellular signaling. Screening experiments using the Biotin Switch Method and modifications thereof have presented the field with a large number of potential target proteins. The present challenge to the field is to determine whether S-nitrosated proteins present in the cell play a meaningful role in signaling or are an artifact of altered redox status. The techniques mentioned above, if carefully applied, may answer some of the outstanding questions regarding nitric oxide-mediated post-translations protein modifications as a true signaling pathway analogous to protein phosphorylation.

APPENDIX A: NITRIC OXIDE DECREASES THE SENSITIVITY OF PULMONARY ENDOTHELIAL CELLS TO LPS-INDUCED APOPTOSIS IN A ZINC DEPENDENT FASHION

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Tang, Z., Wasserloos, K. J., Liu, X., Stitt, M. S., Reynolds, I. J., Pitt, B. R., and St. Croix, C. M. Nitric oxide decreases the sensitivity of pulmonary endothelial cells to LPS-induced apoptosis in a zinc-dependent fashion. Mol. Cell. Biochem. 2002: **234/235**: 211-217.

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A.1.1 Abstract

We hypothesized that: (a) S-nitrosylation of metallothionein (MT) is a component of pulmonary endothelial cell nitric oxide (NO) signaling that is associated with an increase in labile zinc; and (b) NO mediated increases in labile zinc in turn reduce the sensitivity of pulmonary endothelium to LPS-induced apoptosis. We used micro spectrofluorometric techniques to show that exposing mouse lung endothelial cells (MLEC) to the NO-donor, S-nitrosocysteine, resulted in a 45% increase in fluorescence of the Zn^{2+}-specific fluorophore, Zinquin, that was rapidly reversed by exposure to the Zn^{2+} chelator, NNN'N'-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN). The absence of a NO-mediated increase in labile Zn^{2+} in MLEC from MT-I and MT-II knockout mice inferred a critical role for MT in the regulation of Zn^{2+} homeostasis by NO. Furthermore, we found that prior exposure of cultured endothelial cells from sheep pulmonary artery (SPAEC), to the NO-donor, S-nitroso-N-acetylpenicillamine (SNAP) reduced their sensitivity to lipopolysaccharide (LPS) induced apoptosis. The anti-apoptotic effects of NO were significantly inhibited by Zn^{2+} chelation with low doses of TPEN (10 μM). Collectively, these data suggest that S-nitrosylation of MT is associated with an increase in labile (TPEN chelatable) zinc and NO-mediated MT dependent zinc release is associated with reduced sensitivity to LPS-induced apoptosis in pulmonary endothelium (Mol Cell Biochem **234/235**: 211-217, 2002)

A.1.2 Keywords

Live cell imaging

Zinquin

S-nitrosylation

A.1.3 Introduction

Nitric oxide (NO) is a diffusible, multifaceted trans-cellular messenger that is recognized to exert its influence on a myriad of biological functions in a guanylyl cyclase-dependent fashion. However, it has become increasingly apparent that post-translational modifications of proteins, in a guanylyl cyclase-independent fashion, may also contribute to the diverse actions of NO. Covalent attachment of NO groups to protein sulphydryl groups is one such important post-translational modification and over 100 candidates for S-nitrosylation have now been identified [159]. We have recently shown that S-nitrosylation of zinc thiolate clusters in the metal-binding protein, metallothionein (MT), is a crucial component of cellular redox sensitivity linking NO to zinc homeostasis in pulmonary endothelial cells [60; 62].

Recent data shows that NO can act as an anti-inflammatory cyto-protective molecule in a number of different cell types [154]. Endogenous NO synthesis or exposure to low levels of NO donors reduces the sensitivity of cultured endothelial cells to lipopolysaccharide (LPS) induced apoptosis [23; 26]. Although the mechanism remains unclear, in other cell types, the anti-apoptotic effects of NO appear to be associated with increased expression of stress genes such as heat shock proteins [236] and BCL-2 [237], changes in metal (Fe) ion homeostasis [236] and/or guanylyl cyclase-independent modification of Fas ligand signaling pathways by S-nitrosylation

of caspases [238; 239]. We propose that a novel intracellular signaling pathway, S-nitrosylation of zinc-thiolate clusters, may contribute to the NO-mediated inhibition of LPS-induced apoptosis in cultured endothelial cells. In this regard, zinc, the first known inhibitor of apoptosis [240], may be a candidate molecule in mediating the sensitivity of endothelial cells to pro-apoptotic stimuli after NO. This hypothesis is based on separate reports showing that exposure to NO donors increases intracellular zinc [59; 62; 135] and that Zn^{2+} chelation enhances LPS-induced apoptosis in cultured endothelial cells [120]. We have also shown that the major zinc binding protein, metallothionein, is required for NO-mediated zinc release in cultured fibroblasts [62]. However, given that endothelium is a critical locus of the L-arginine-NO biosynthetic pathway, and has been the focus of early structural and functional changes in a variety of pro-oxidative conditions, many of which are affected by the simultaneous production of nitric oxide, it was important to determine the importance of MT in affecting changes in labile zinc in endothelial cells, in addition to investigating a role for such alterations in intracellular Zn^{2+} in the anti-apoptotic actions of NO.

In the present communication, we used live cell imaging of cultured endothelial cells isolated from the lungs of wildtype (MT +/+) and MT null mutant mice (MT -/-) to show that MT plays a central role in mediating NO-induced changes in labile Zn^{2+} in pulmonary endothelium. Furthermore, we demonstrated that NO-mediated inhibition of apoptosis is reversed with the Zn^{2+} chelator TPEN, suggesting that NO-induced release of Zn^{2+} from MT could be a contributing factor underlying the anti-apoptotic effects of NO.

A.1.4 Materials and methods

A.1.5 MT knockout mice

Breeding pairs of MT-I and MT-II deficient mice (MT-/-) were imported from Michalska and Choo [241]. The mice were of a mixed genetic background of OLA129 and C57BL6 strains. To dilute the OLA129 genetic contribution, MT -/- mice were bred with C57BL6 mice obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA) to generate a parental heterozygous chimera that in turn was backbred to C57BL6 wild type. This backbreeding resulted in ≈ 50% offspring that were heterozygous mutants. These mutants were identified through a genotyping protocol using polymerase chain reaction strategy on novel sites within the murine MT-II gene that was mutated. Additional rounds of interbreeding and genotyping resulted in F2 generation of MT +/+ and MT -/- mice, which allowed the establishment of breeding colonies in which the genetic contributions of the two strains were assumed to be similar.

A.1.6 Cultured murine lung endothelial cells

MT +/+ and MT -/- endothelial cells were isolated by modifications of an immunobead protocol [242]. Briefly, mice lungs were rinsed in PBS, finely minced and digested in collagenase (Type I, 100 μg/ml) for 60 minutes at 37°C with occasional agitation. The mixture was filtered through 100 μm cell strainer, centrifuged and washed twice in medium. Cell suspensions were incubated with a monoclonal antibody (rat anti-mouse) to platelet endothelial cell adhesion molecule-1 (PECAM-1, BD Pharminigen, San Diego, CA, USA) for 30 minutes at 4°C. Constitutive

expression of PECAM-1 is a fundamental characteristic of endothelial cells and has been used extensively as a reliable marker to isolate endothelial cells [242]. The cells were washed twice with buffer to remove unbound antibody, and resuspended in binding buffer containing the appropriate number of washed magnetic beads coated with sheep anti-rat IgG (Dynal, Oslo, Norway) to give the desired 30:1 bead to cell ratio. Attached cells were washed 4-5 times in cell culture medium, and then were digested with trypsin/EDTA to detach the beads. Bead-free cells were centrifuged and resuspended for culture. At approximately passage 2, cells were incubated with fluorescent-labeled di-acetylated LDL (dil-LDL), which is taken up only by endothelial cells and macrophages, and sorted to homogeneity by FACS. The enriched PECAM and dil-LDL population were subcultured in DMEM/F-12, 20% fetal bovine serum, 6% plasma-derived human serum, 2 mM glutamine, and 30 μg/ml endothelial derived growth factor.

A.1.7 Cultured sheep pulmonary artery endothelial cells (SPAEC)

SPAEC were cultured from sheep pulmonary arteries obtained from a nearby slaughterhouse as previously described [128] and grown in OptiMEM (GIBCO BRL, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2.

A.1.8 Fluorescent microscopy

MT +/+ and MT -/- endothelial cells were plated onto 31 mm glass coverslips (VWR Scientific, West Chester, PA, USA). Cells were washed with PBS and incubated with 15-30 μM Zinquin (Toronto Research Chemicals, Toronto, ON, Canada) for 20 minutes at 37°C. All recording

were performed at room temperature (20-25°C). Cells were imaged using a PC-based system consisting of a Nikon Diaphot 300 microscope equipped with a quartz 40X oil immersion objective, a CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan), SimplePCI software from Compix (Cranberry, PA, USA) and a monochromator-driven xenon light source (ASI, Eugene, OR, USA). Zinquin was illuminated at 350 nm; light was passed through a400 nm dichromatic mirror, and emitted fluorescence was filtered through a 510 ± 40 nm bandpass emission filter (Omega Optical, Brattleboro, VT, USA). For analysis of images, background illumination was subtracted from the readings and fluorescent intensity was expressed relative to baseline measurements. Cells were exposed to the NO donor S-nitrosocysteine (SNOC, 2 mM) and time-dependent changes in Zinquin fluorescence were monitored as an index of labile Zn^{2+}., Cells were also exposed to 100 μM Zn^{2+} (in the presence of 10 μM pyrithione) and the zinc-specific chelator N, N, N', N'-tetrakis (2-pyridylmethyl)ethylenediamine (TPEN, Sigma, St. Louis, MO, USA) as positive controls and denitrosylated SNOC for negative controls. Each experiment was repeated on separate coverslips a minimun of 3 times per cell type.

A.1.9 Lipopolysaccharide (LPS)-induced apoptosis

SPAEC were exposed to 0.1 μg/ml LPS (*Escherichia coli* 0111:B4, Sigma) for 12 h and apoptosis was quantified by Hoechst 33342 (1 μg/ml; Molecular Probes, Eugene, OR, USA) fluorescent staining [243]. The sensitivity of SPAEC to LPS was reassessed following pre-treatment with the NO-donor S-nitroso-N-acetylpenicillamine (SNAP; Sigma, St. Louis, MO,

USA; 500 μM) for 6 h prior to and during LPS exposure. The contribution of Zn to NO mediated LPS resistance was then determined by repeating this latter experiment in the presence of TPEN (10 μM).

A.1.10 NO-induced cytotoxicity

SPAEC were incubated 1-10 μM SNAP for 24 h or 0-10 mM SNOC for 2 h. Toxicity was assessed by incubating the cells with the oxidized form of the fluorogenic compound Alamar Blue (Accumed, Chicago, IL, USA) and determining the change in fluorescence emission due to cellular reduction via mitochondrial respiration as previously described [244].

A.2 RESULTS

A.2.1 Effects of NO on zinc homeostasis in endothelial cells

We used the Zn^{2+}-specific fluorophore, Zinquin [245; 246] to image labile Zn^{2+}. As reported in other cell types [62; 135; 247], lung endothelial cells isolated from both MT +/+ and MT -/- mice showed discrete areas of strong fluorescence within the cytoplasm (Fig. A.1).

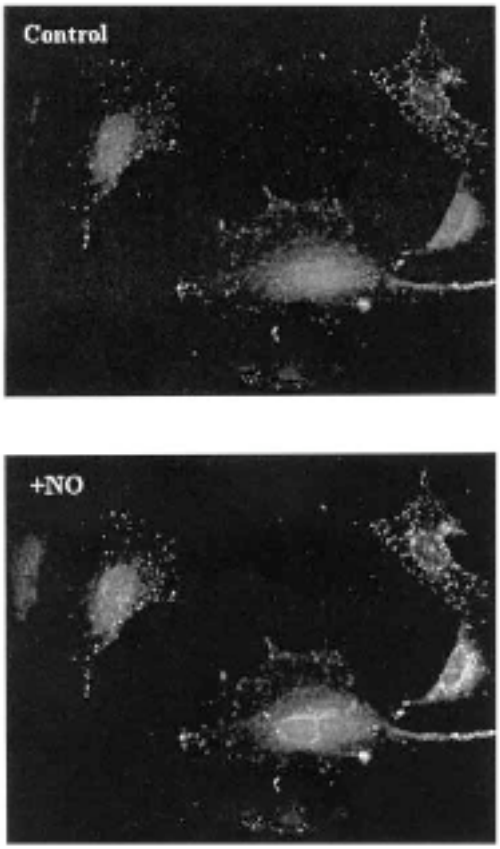

Figure A 1 Zinquin fluorescence measured in mouse lung endothelial cells. Wild-type (MT +/+) cells under control conditions (top panel) and following exposure to 2 mM SNOC (bottom panel).

Unlike pulmonary artery endothelial cells isolated from sheep, or mouse lung fibroblasts [62], mouse lung endothelial cells also showed low amounts of diffuse fluorescence in the nucleus. When MT +/+ were exposed to 2 mM SNOC there was a prompt and gradual increase in fluorescence above baseline levels (Figs A.1 and A.2) that returned to levels below control with the application of the Zn^{2+}-chelator, TPEN (Fig. A.2A).

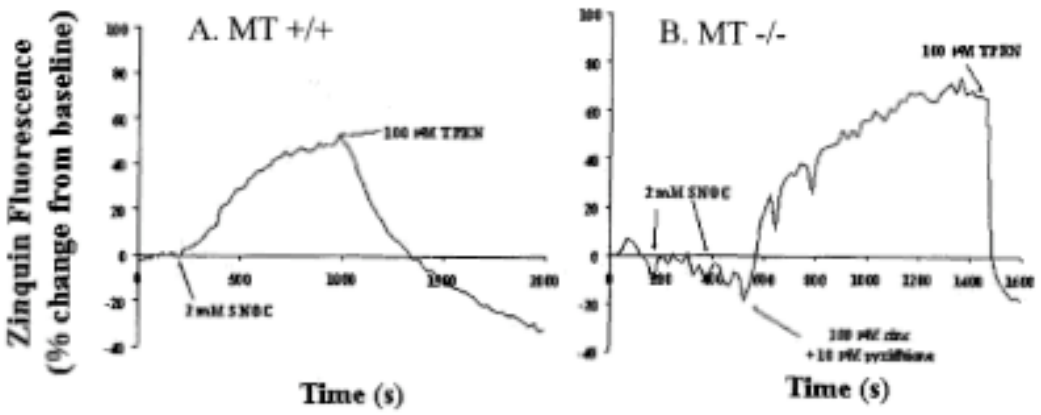

Figure A 2 Zinquin fluorescence in lung endothelial cells from MT +/+ and MT -/- mice. S-nitrosocystein (SNOC, 2 mM) increased Zinquin fluorescence in lung endothelial cells isolated from MT +/+ mice (A), but had no effect in endothelial cells from MT -/- mice (B). Each experiment is represented by a single trace from multiple cells, and experiments were repeared 3 or 4 times per condition.

This response was consistent in 3 separate experiments (approximately 3-5 cells per experiment) and averaged a 44.9 ± 3.1% (SD) increase above baseline fluorescence in response to SNOC. In contrast, there was no increase in fluorescent intensity in response to repeated applications of 2 mM SNOC (> 10 cells, mean change = 8.2 ± 2.1%) in endothelial cells from MT knockout mice (Fig. A.2B) suggesting that MT was central to the changes in labile Zn^{2+} observed in MT +/+.

A.2.2 Effects of NO on LPS-induced apoptosis in endothelial cells

As previously reported [23; 120], exposure of endothelial cells to 0.1 μg/ml LPS (12 h) resulted in a significant increase in apoptosis (Fig A.3).

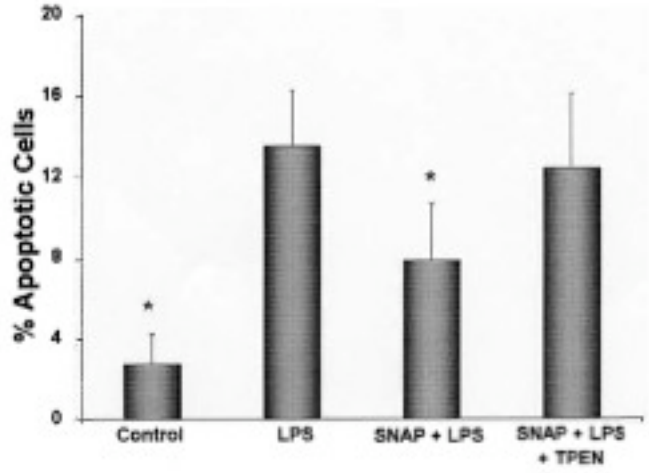

Figure A 3 Apoptosis in SPAEC exposed to LPS. Sheep pulmonary artery endothelial cells (SPAEC) were exposed to 0.1 μg/ml LPS for 12 h and apoptosis determined by Hoescht 33342 staining and fluorescence chromatin determination and quantification. The protective effects of NO on LPS-induced apoptosis were assessed by pre-treatment with 500 μM SNAP. This protection was reversed by the application of 10 μM TPEN. * indicate a significant difference from LPS alone ($P<0.05$).

However, SPAEC that were pretreated with the NO donor, SNAP (500 μM) showed decreased sensitivity to LPS-induced apoptosis. This protective effect of NO was significantly inhibited by zinc chelation with TPEN (10 μM). Exposure to this low level of TPEN is sufficient to decrease labile zinc [120] but is not significantly toxic to SPAEC. However, higher concentrations of TPEN (> 10 μM) were previously shown to induce a concentration-dependent decrease in viability and increase in apoptosis in pulmonary endothelial cells [120].

While high concentrations of NO donors are toxic in other cell types, a 24 h exposure to 500 μM SNAP did not affect the viability of cultured SPAEC (Fig. A.4).

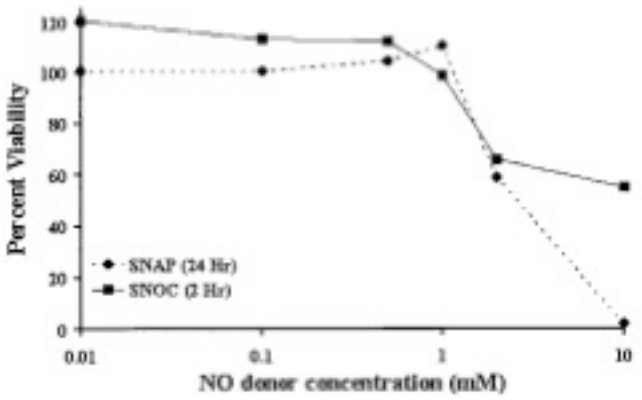

Figure A 4 Cytotoxicity of NO donors in SPAEC. Sheep pulmonary endothelial cells (SPAEC) were incubated with 0-10 mM SNAP for 24 h or 0-10 mM SNOC for 2 h and cell viability was assessed with Alamar Blue.

We also examined the sensitivity of SPAEC to SNOC. Given the short half-life of this NO donor (~ 3 min), we assessed cytotoxicity after 2 h and showed that endothelial cells were remarkably resistant to high levels of SNOC with only a 30-40% decrease in cell viability at concentrations in excess of 2 mM.

A.3 DISCUSSION

Recent evidence suggests that S-nitrosylation of zinc sulfur clusters is an important component of NO signaling [59; 60; 62; 248] and our present data confirms that metallothionein is a critical link between NO and intracellular zinc homeostasis in pulmonary endothelium. Furthermore, we found that exposure of endothelial cells to low doses of NO reduced their sensitivity to subsequent LPS exposure and showed that these anti-apoptotic effects of NO were sensitive to Zn^{2+} chelation by TPEN. Collectively, these data suggest that MT is a critical compartment for

labile Zn^{2+} and this function is important for the physiologic roles of both MT and NO in protecting the cell from oxidative stress.

A.3.1 Nitric oxide and apoptosis

The NO donors S-nitrosocysteine (SNOC) and S-nitroso-N-acetylpenicillamine (SNAP) spontaneously decompose to generate NO and disulfide. SNOC decays with a half-life in the range of 2-3 min whereas SNAP has a longer half-life on the order of several hours [248]. While the concentrations of SNOC (2 mM) or SNAP (500 μM) used in these experiments appear relatively high, measurements made of the rate of decomposition of these donors [151] and/or free NO concentrations [249; 250] suggest that the [NO] generated by either SNOC or SNAP is actually 2-3 orders of magnitude less than the donor concentration. In addition, the release kinetics of SNOC suggest that cell cultures are exposed to a burst of NO, most of which is auto-oxidized or has reacted with media components before reaching the target cells at the bottom of the culture dish [134]. We would therefore expect the actual [NO] to be much closer to physiological levels reported to be in the nanomolar range under resting conditions [251] and as high as 1.3 μM following stimulation with bradykinin, as measured directly with phorphyrinic sensors positioned *in situ* at the endothelial surface of rabbit aorta [252].

High concentrations of NO donors have been shown to induce apopotosis in a number of cell types, including macrophages, pancreatic islets and certain neurons [154]. In other cell types, even low levels of NO can cause necrotic cell death [253; 254]. However, we have shown that pulmonary endothelial cells are remarkably resistant to NO-induce cytotoxicity. In general, if a cell is depleted of GSH and is undergoing oxidative stress, then exposure to large amounts of NO invariably leads to cell death. In contrast, in a cell with a more favorable redox status,

physiologically relevant levels of NO have been shown to suppress apoptosis in a variety of cell types, induced by a number of stimuli, including TNF, oxidative stress and growth factor withdrawal [154]. This effect may be guanylyl cyclase dependent or independent. In the latter case, NO appears capable of inducing stress genes that account for decreased apoptosis [236]. NO can also nitrosylate caspase-3 and thus interfere with the apoptotic pathway [239; 255]. This phenomenon was originally noted in cultured endothelial cells that became resistant to LPS-induced apoptosis when synthesizing NO after direct gene transfer of human inducible nitric oxide synthase (iNOS) [23; 26]. In the present study, the anti-apoptotic effects of NO were TPEN-sensitive, suggesting that NO-mediated resistance to LPS involved zinc itself or zinc-dependent downstream targets.

A.3.2 Measurement of labile zinc

After iron, zinc is the major intracellular metal, but its free concentration is maintained at extraordinarily low levels by the actions of various zinc transporters, vesicular storage sites and metal binding proteins of which MT is a major contributor. Nevertheless, it is this labile pool of Zn^{2+} that appears to be critical in affecting various cellular processes. Zinquin ester [245; 246] is a quinoline-based, non-fluorescent, membrane permeable fluorophore that becomes strongly fluorescent upon specific binding to Zn^{2+} and can thus be used to assess the intracellular disposition of chelatable Zn^{2+} with detection limits that range from 4 pM to 100 nM [256].

The present data confirmed that NO-induced increases in labile Zn^{2+} are dependent upon metallothionein in pulmonary endothelial cells, as shown previously in fibroblasts [62], and suggested by the NO-induced conformational changes in green fluorescent protein modified metallothionein [60]. In vitro studies using raman spectroscopy suggest that the NO generated

by SNOC mediates Zn^{2+} release from MT via nitrosylation of cysteine thiol groups and subsequent disulfide formation [59].

A.3.3 Zinc and apoptosis in pulmonary endothelium

Recent studies using Zinquin revealed labile pools of zinc in pulmonary endothelium [120] and epithelium [247] and chelation of Zn^{2+} resulted in spontaneous apoptosis in both cell types. We recently reported that, like many other cell types, including systemic endothelial cells [257; 258; 259; 260], zinc chelation enhanced LPS-induced apoptosis [120]. We now show that chelation of Zn^{2+} by TPEN inhibited the anti-apoptotic effects of NO pre-exposure in pulmonary endothelial cells. These data are consistent with the findings of Hennig [259; 260; 261] and others [262; 263] who have shown that culturing systemic endothelial cells in zinc depleted medium or exposing these cells to TPEN enhanced their sensitivity to various pro-apoptotic stimuli. For example, in cultured aortic endothelial cells, zinc can block cadmium- [262], cholesterol- [264] and linoleic acid and TNF-alpha [261] induced apoptosis. The results are not surprising given the fact that zinc was the first molecule shown to inhibit apoptosis. It was initially thought that Zn^{2+} directly inhibited Ca^{2+}/Mg^{2+}-dependent endonucleases [265]. More recent data suggests that zinc affects the activity [266] or processing [267; 268] of caspase-3, a critical protease in apoptosis. Zn^{2+} itself is redox inert, but it is associated with an extraordinary large number of proteins (estimated between 1-10% of the human genome) and is thus an integral component of numerous metalloenzymes, structural proteins, and transcription factors. As such, it is highly likely that there are alternative pathways mediating the zinc-dependent, anti-apoptotic effects of NO that could even involve DNA synthesis and the expression of protective

genes. Future work should be directed towards identification of the critical targets for zinc following NO-induced release from MT.

A.4 ACKNOWLEDGEMENTS

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APPENDIX B: NITRIC OXIDE-INDUCED MODIFICATION OF PROTEIN THIOLATE CLUSTERS AS DETERMINED BY SPECTRAL FLUORESCENCE RESONANCE ENERGY TRANSFER IN LIVE ENDOTHELIAL CELLS

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St. Croix, CM., Stitt, MS., Leelavanichkul, K., Wasserloos, KJ., Pitt, BP., and Watkins, SC. Nitric oxide-induced modification of protein thiolate clusters as determined by spectral fluorescenc resonance energy transfer in live endothelial cells. *Free Rad. Biol. Med.* **37**: 785-792, 2004.

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B.1.1 Abstract

Low-molecular-weight S-nitrosothiols are found in many tissues and affect a diverse array of signaling pathways via decomposition to •NO or exchange of their –NO function with thiol-containing proteins (transnitrosation). We used spectral laser scanning confocal imaging to visualize the effects of D- and L-stereoisomers of S-nitrosocysteine ethyl ester (SNCEE) on fluorescence resonance energy transfer (FRET)-based reporters that are targets for the following NO-related modifications: (a) S-nitrosation, via the cysteine-rich protein metallothionein (FRET-MT), and (b) nitrosyl–heme–Fe, via guanosine 3',5'-cyclic monophosphate (cygnet-2). Conformational changes consistent with S-nitrosation of FRET-MT were specific to L-SNCEE. In addition, they were reversed by dithiothreitol (DTT) but unaffected by exogenous oxyhemoglobin. In contrast, D- and L-SNCEE had comparable effects on cygnet-2, likely via activation of soluble guanylyl cyclase (sGC) by •NO as they were sensitive to the sGC inhibitor 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one and exogenous oxyhemoglobin. These data

demonstrate the utility of spectral laser scanning confocal imaging in revealing subtle aspects of NO signal transduction in live cells. Stereoselective transnitrosation of MT emphasizes the specificity of posttranslational modification as a component of NO signaling.

B.1.2 Keywords

Nitrosothiol, Metallothionein, Guanosine 3',5'-cyclic monophosphate, Live cell imaging, Green fluorescent protein, S-nitrosation, Free radicals

B.1.3 Introduction

S-nitrosothiols are adducts of nitric oxide (•NO) and thiol-containing compounds that are found in many mammalian tissues and are suggested to affect a diverse array of physiological functions, including signal transduction and immune responses [269]. The bioactivities of low-molecular-weight S-nitrosothiols may be mediated either by their decomposition to •NO or by direct exchange of their –NO function with thiol-containingproteins (transnitrosation) [34]. Detection of interactions between target proteins and nitrogen oxide species in vivo typically requires disruptive biochemical techniques that preclude or limit temporal–spatial information. Although significant technological advancements have promoted an increased reliance on the use of high performance multimodal optical imaging tools to visualize cellular processes in biological systems, the imaging of NO-based signaling events remains especially challenging. One successful example is the use of spatial–spectral electron paramagnetic resonance imaging, in combination with isotope tracer methods, to map nitrite-derived NO production in the isolated ischemic rat heart [270]. More recently, the fluorescent indicator 4,5-diaminofluorescein [271;

272] has been widely used for detectionand imaging of •NO but is hampered by concerns regarding its selectivity [273; 274], sensitivity [273], and interactions with divalent cations [275].

Fluorescence resonance energy transfer (FRET) is a nondestructive spectrofluorometric technique. In combination with scanning laser confocal microscopy, FRET is capable of detecting changes in the conformational state of proteins in live cells. The validity of FRETbased approaches to studying intracellular signaling pathways in live cells has been demonstrated using fluorescent reporter molecules for calcium [276], guanosine 3',5'-cyclic monophosphate (cGMP) [277], 3',5'cyclic adenosine monophosphate [278], and tyrosine kinase activity [279], among others. Our recent efforts utilizing this methodology to elucidate the role of metallothioneins (MT) in nitric oxide signaling [60; 62] suggest that FRET is suitable for detection of posttranslational protein modifications caused by NO-related species.

FRET is a physicochemical phenomenon whereby two fluorophores (i.e., donor and acceptor pair) that have appropriate spectral properties and are closely apposed (less than 10 nm) transfer photoelectric energy in a nonradiative fashion. Established methods for detecting FRET between a donor and an acceptor with overlapping excitation and emission spectra require the use of narrow detection bands and automatic switching of optical filters to differentiate between emissions, along with complex mathematical corrections to account for cross talk between channels. Recent advances in detector technology, however, now enable the resolution of fluorescent images providing full spectral information for each voxel of the image without switching of optical filters. Furthermore, using calibration spectra, it is possible to unambiguously separate the cross talk between overlapping cyan and yellow emissions. The use of this method allows the detection of small, but potentially biologically meaningful, changes in FRET that are common with genetically encoded reporters and are extremely difficult to resolve

reliably using more traditional methods relying on bandpass filters.

We used this spectral resolution approach to visualize the effects of membrane-permeant forms (ethyl esters) of the D- and L-stereoisomers of S-nitrosocysteine on FRET-based reporters for cGMP (cygnet-2) [277] and for the cysteine-rich, metal-binding protein metallothionein (FRET-MT). We hypothesized that different effects of SNCEE on the two FRET reporters would distinguish between signaling events that are mediated by the free radical •NO, as is best exemplified by the activation of soluble guanylyl cyclase (sGC) and direct transnitrosation of cysteine thiols. Furthermore, reports showing that the cardiovascular [280; 281] and ventilatory [196] effects of S-nitrosocysteine are specific to the L-isoform suggest that transnitrosation may be stereoselective, as a consequence of the quaternary structure of target peptides [282].

B.1.4 Experimental Methods

B.1.5 Cultured sheep pulmonary artery endothelial cells

SPAEC were cultured from sheep pulmonary arteries obtained from a nearby slaughterhouse as previously described [128]. The SPAEC were grown in OptiMEM (GIBCO) supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere with 5% CO_2.

B.1.6 FRET-MT

We followed the example of the Ca2+ indicator cameleon-1 [276] and constructed a chimera in which a yellow green fluorescent protein (GFP) variant (EYFP) and a cyan GFP variant (ECFP)

were fused to the COOH and NH2 termini, respectively, of human MTIIA (FRET-MT) [60]. An E1- and E3-deleted replication- deficient adenoviral vector expressing this chimera was constructed as previously described [62] and cells were infected at a multiplicity of infection of 50:1 for 24 h before imaging.

B.1.7 Cyclic GMP reporter

The cDNA encoding a fluorescent cGMP indicator consisting of cGMP protein kinase G Ia (PKGIa), minus residues 1–77, bracketed between cyan and yellow mutants of green fluorescent protein (cygnet-2) [277] was kindly provided by Roger Y. Tsien (University of California at San Diego). This reporter can detect changes in the activation of guanylyl cyclase indirectly via structural changes in cGMP-dependent protein kinase [277]. SPAEC that expressed cygnet-2 were imaged 48 h after transfection with Lipofectamine Plus (Gibco BRL).

B.1.8 Preparation of reagents

The hydrochloride of S-nitroso-L-cysteine ethyl ester was prepared via direct S-nitrosation of the hydrochloride of L-cysteine ethyl ester with ethyl nitrite, as previously described [127]. D-Cysteine ethyl ester was prepared as described by Baldwin [283]. Briefly, D-cysteine hydrochloride monohydrate was dissolved in dry distilled methanol, and HCl gas was bubbled through the solution for 2 h and then stirred at room temperature (48 h). The salient was rotor-evaporated at 20°C to yield an oil which, upon titration with diethyl ether, yielded D-EtO-Cys-SH (99%) as white crystals. The product's purity was controlled by determination of free SH groups with 5,5'-dithiobis-2-nitrobenzoic acid [284] and by HPLC-EC [62]. Human hemoglobin

(Sigma–Aldrich) was dissolved in Hanks' balanced salt solution (Gibco BRL) and reduced using a 10-fold excess of sodium ascorbate (Sigma–Aldrich). Oxyhemoglobin (HbO_2) was purified by passing the solution over a Sephadex G-25 column and the concentration of HbO_2 was measured at an absorbance of

415 nm. Final concentrations of 100 μM HbO_2 were used in the described experiments.

B.1.9 Determination of nitrite concentration

Two separate subcultures of SPAEC were exposed to 200 μM D- or L-SNCEE for 2 h. Nitrite accumulation in the culture supernatant was assayed in triplicate with a Greiss reaction [285]. Absorbance was measured at 550 nm and nitrite was quantified with $NaNO_2$ as a standard.

B.1.10 FRET detection

Cells were bathed in Hanks' balanced salt solution (Gibco BRL) containing 100 μM EDTA to chelate trace amounts of metal ions in the buffer and prevent extracellular decomposition of S-nitrosothiol and imaged at 37°C using a thermocontrolled stage insert (Harvard Apparatus, Inc., Holliston, MA, USA). Images were obtained with a 40x oil immersion optic at 512 x 512 pixels using the confocal-based Zeiss spectral imaging system (LSM510 META; Carl Zeiss, Jena, Germany). Cyan was excited at 458 nm (HFT 458). Resolved fluorescence spectra at each pixel were detected by an array of eight spectrally separate photomultiplier tube elements within the META detection head and recorded on a voxel-by-voxel basis during scanning to generate a set of images, each corresponding to the fluorescence wavelength resolved at 10 nm intervals. Color separation of cyan and yellow emission spectra was determined from the resolved image using a

linear unmixing algorithm based on reference spectra obtained in cells expressing only cyan or yellow protein. Images were collected at baseline and 1, 3, 6, and 9 min after addition of the relevant agonist. In separate control experiments, FRET was confirmed for the FRET-MT and cygnet-2 constructs by acceptor photobleaching (data not shown).

B.2 RESULTS

B.2.1 S-nitrosocysteine ethyl ester activates FRET-MT stereoselectively

SPAEC were infected with an adenovirus encoding the cDNA for a GFP chimeric protein, consisting of the 6 kDa heavy metal binding protein MT, sandwiched between enhanced cyan and yellow fluorescent protein, as previously described [62; 119]. We have shown that this construct is sensitive to a variety of NO donors as well as endothelial nitric oxide synthase-derived NO [62; 119]. We have now extended these findings using the spectral imaging approach for FRET detection, in combination with a novel membrane-permeant S-nitrosothiol, to provide further details regarding the biochemical nature of the nitrosative modifications of MT. Figure B.1 shows a single endothelial cell expressing the FRET-MT reporter after linear unmixing using calibration spectra for ECFP and EYFP.

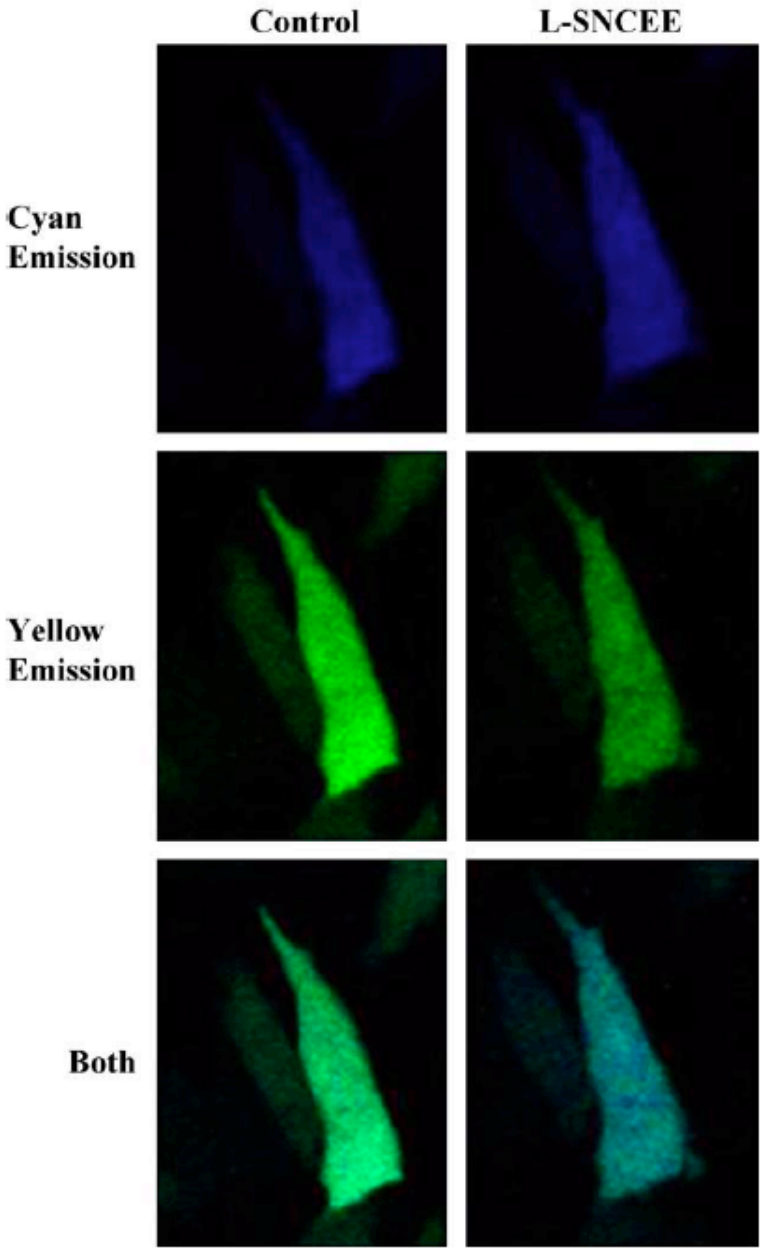

Figure B 1 Cyan and yellow emissions at baseline and after application of L-SNCEE, in live endothelial cells that express the FRET-MT reporter. Lung endothelial cells were infected with an adenoviral vector encoding the fluorescent FRET-MT reporter molecule. FRET was detected in real time, using full spectral confocal imaging. The images illustrate the separation of the two emitted signals (cyan and yellow) after spectral unmixing based on individual calibration spectra for each protein. The accompanying spectral report is represented in Fig. B 2A. After application of L-SNCEE (50 μM) there was an increase in the emission intensity of the donor (cyan) and a decrease in that of the acceptor (yellow). The dual-color image at the bottom highlights this decrease in energy transfer.

These images therefore represent the separate contributions of the cyan and yellow emissions. The spectral report for this cell appears in Fig. B 2A and shows an increase in the peak emission intensity of the donor (cyan, ~485 nm) and a decrease in that of the acceptor (yellow, ~525 nm) after application of L-SNCEE (50 μM).

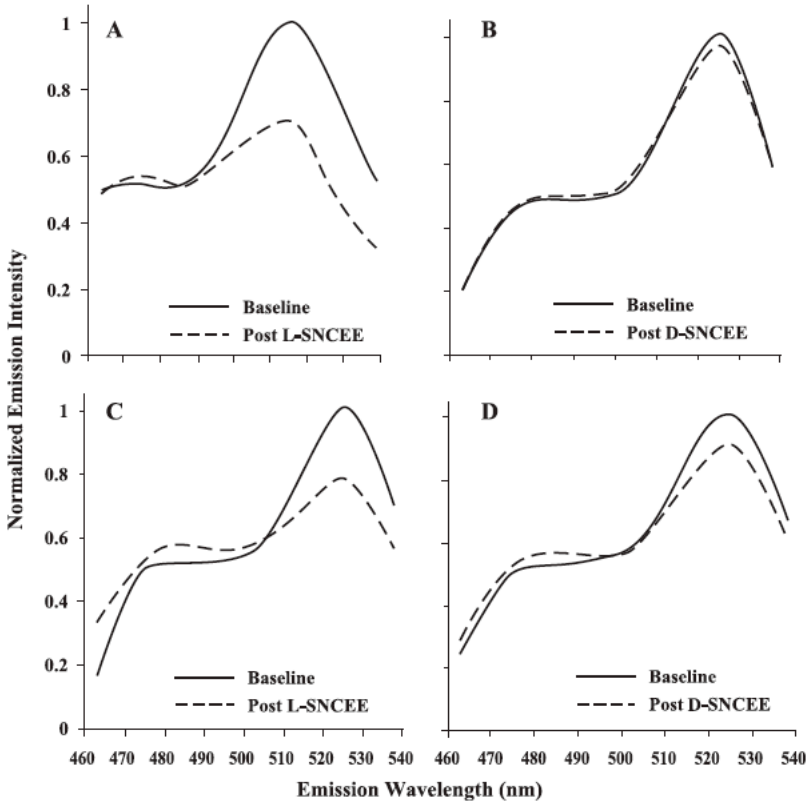

Figure B 2 Spectral reports for individual endothelial cells expressing (A and B) FRET-MT or (C and D) cygnet-2. L-SNCEE (50 μM) induced conformational changes in the FRET-MT reporter as shown by a decrease in energy transfer with an increase in the peak emission intensity of the donor (cyan, ~485nm) and a decrease in that of the acceptor (yellow, ~525nm) (A), whereas D-SNCEE (50 μM) had no appreciable effect on the FRET-MT reporter molecule (B). In contrast, both L-SNCEE (C) and D-SNCEE (D) induced conformational changes in the cygnet-2 (cGMP) reporter.

In three subcultures of SPAEC expressing the FRET-MT reporter (three to five cells per experiment) the intramolecular FRET ratio was decreased on average by 34.1 ± 4.0% (SD) in response to L-SNCEE (Fig B 3).

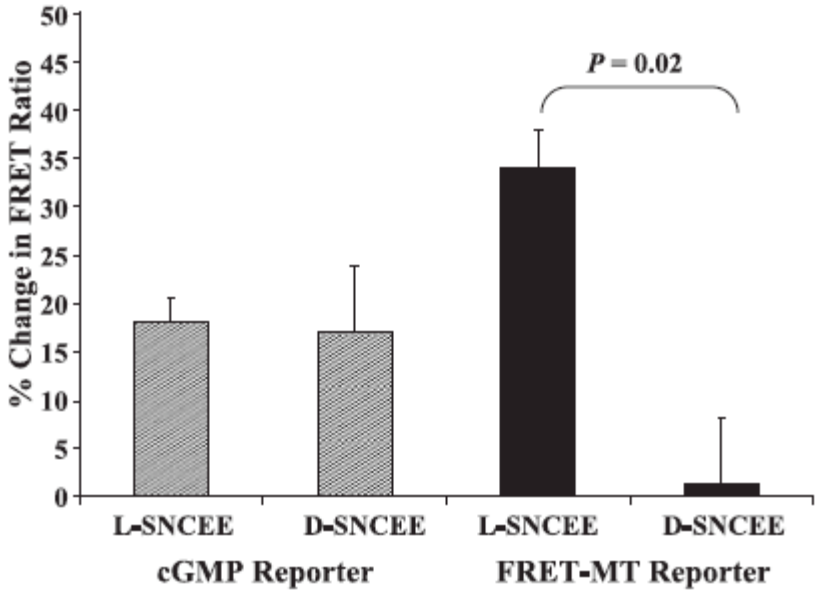

Figure B 3 Mean changes (± SD) in FRET, expressed as a percentage change from control, for both the cgnet-2 (cGMP) and the FRET-MT reporter molecules in response to the L- and D-stereoisomers of SNCEE. The mean represents three experiments per condition (three to five cells per experiment).

In separate experiments L-SNCEE-induced changes in FRET were reversed by 50 μM dithiothreitol (DTT; Fig.B 4).

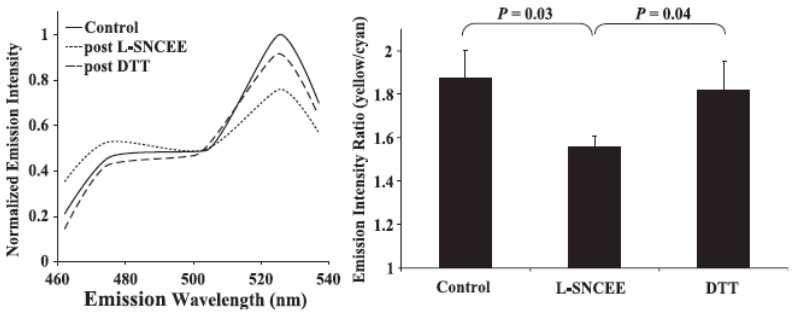

Figure B 4 The effects of L-SNCEE on FRET-MT were reversed by dithiothreitol (DTT). As shown by the spectral report from a single cell (left), L-SNCEE (50 μM) caused a decrease in the peak emission of the FRET donor (cyan, 458 nm) and an increase in the peak emission of the FRET acceptor (yellow, 525 nm). Application of DTT (50 μM) reversed these effects. Mean data (right) from seven cells in three separate experiments showed a significant decrease in FRET ratio (from 1.9 ±0.1 to 1.5 ± 0.1, $p<0.05$) after application of L-SNCEE and an increase in FRET ratio to baseline levels (1.8 ± 0.1, $p>0.05$) 6 min after DTT treatment.

Such reversibility is suggestive of nitrosothiol-mediated modification of a cysteine residue(s) in MT. Furthermore, the effects of L-SNCEE on the FRET-MT reporter were not affected by HbO_2 (Fig. B 5), which would be expected to block any process involving •NO liberated by decomposition of nitrosothiol, but would not necessarily affect transnitrosation.

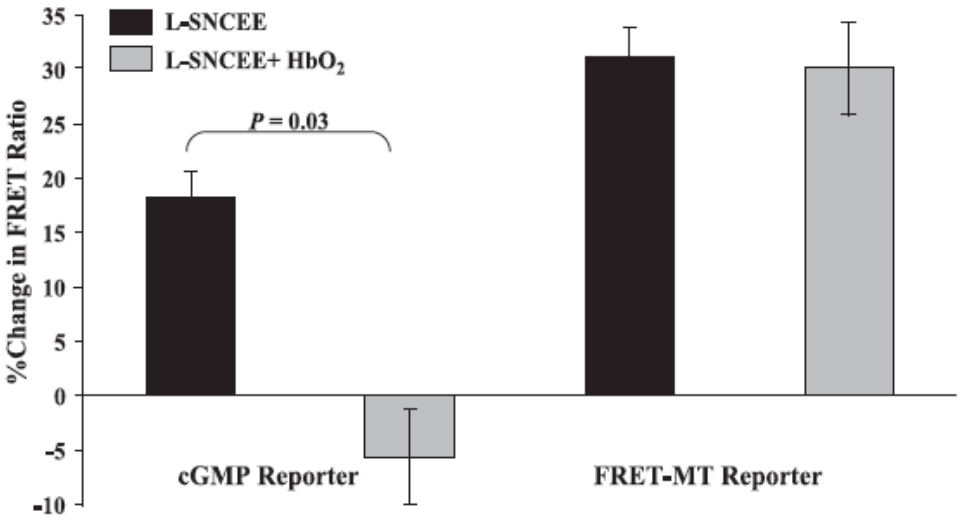

Figure B 5 The effects of L-SNCEE on FRET-MT were not affected by oxyhemoglobin. Mean changes (± SD) in FRET, expressed as a percentage change from control, for both the cygnet-2 (cGMP) and the FRET-MT reporter molecules in response to L-SNCEE in the presence or absence of oxyhemoglobin (100 μM). The mean represents three experiments per condition (three to five cells per experiment).

In contrast to the effects of L-SNCEE on FRET-MT, there was no significant change in FRET in response to equimolar concentrations of D-SNCEE (Figs. B 2B and B 3). D- and L-SNCEE decomposed equally in cell-based assays, as determined by the accumulation of nitrite in EDTA-free culture medium. In addition, both isoforms activated protein kinase G Ia to similar extents (Fig. B 3, see below), suggesting that the stereoselective effect was specific to MT. These combined results most likely reflected the trans-S-nitrosation of thiolate clusters of MT by L-SNCEE.

B.2.2 S-nitrosocysteine ethyl ester activates the cGMP reporter cygnet

SPAEC were transfected with cDNA encoding a fluorescent cGMP indicator (cygnet-2) consisting of cGMP-dependent PKGIa, minus residues 1–77, bracketed between cyan and yellow mutants of green fluorescent protein (kindly donated by Roger Y. Tsien, University of California at San Diego). cGMP induces conformational changes in PKGIa, detectable as decreases in FRET between the cyan and the yellow protein with the cygnet-2 reporter [277]. As shown in Fig. B 2C, L-SNCEE caused an increase in the peak emission intensity of the donor (cyan) and am decrease in that of the acceptor (yellow). This decrease in energy transfer was consistent with increases in cGMP, as previously shown with this FRET reporter in response to activation of soluble or particulate guanylyl cyclase by nitric oxide donors or C-type natriuretic peptide, respectively [277]. In contrast to the FRET-MT reporter molecule, cygnet-2 responded equally to both the L- and the D-stereoisoforms of S-nitrosocysteine, with decreases in the FRET ratio of 18.1 ± 3.6 and 17.0 ± 7.0%, respectively (Figs. B 2D and B 3). A 10 min pretreatment of cells with 10 MM ODQ (1H-[1,2,4]-oxadiazolo[4,3-a] quinoxalin-1-one), a selective inhibitor of sGC, prevented the SNCEE-mediated changes in energy transfer (mean change in FRET ratio, 1.5 ±10.4%), demonstrating that these effects were dependent upon activation of sGC and resultant increases in cGMP. Cygnet-2 also responded effectively to the •NO donor PAPAnonate (100 μM), with a mean decrease of 13.9 ± 1.2% in energy transfer.

B.3 DISCUSSION

We observed (Figs. B 2A, B 2B, and B 3) stereospecific effects of the membrane-permeant forms of the D- and L-isoforms of SNCEE on the FRET-MT reporter. D- and L-SNCEE decomposed equally in cell-based assays, however, and effected comparable changes in the cGMP reporter cygnet-2, likely via activation of soluble guanylyl cyclase by formation of an Fe–nitrosyl heme complex. L-SNCEE-induced changes in FRET-MT were reversible by DTT (Fig. B 4) and insensitive to extracellular oxyhemoglobin (Fig. B 5). These data corroborate our in vitro finding that NO can S-nitrosate MT [124] and provide spectral laser scanning confocal imaging (Fig.B 1) of FRET-based conformational changes in MT, consistent with direct transnitrosation of the protein.

B.3.1 Transnitrosation of MT by SNCEE

Putative mechanisms governing the stereoselective effects of bolus infusions of S-nitrosocysteine on vascular smooth muscle [280], and on CNS-mediated regulation of cardiovascular [281] and respiratory [196] activities, include stereospecific receptor and/or transporter interactions and stereoselective catabolism. Indeed the mechanisms governing these physiological effects are likely membrane-based because S-nitrosocysteine (SNC) is an unstable, hydrophilic compound that cannot efficiently cross membranes [127]. Many of the biological effects of exogenous application of SNC may therefore be attributed to its extracellular decomposition to •NO. In this report, however, we utilized the D- and L-stereoisomers of the lipophilic SNCEE, previously shown to be stable in solutions containing metal chelators and to accumulate inside human neutrophils [127]. The preferential effects of S-nitroso- L-cysteine on FRET-MT therefore might

suggest that the structure of the L-stereoisomer of SNCEE confers access to a critical cysteine residue(s) in the metallothionein protein and that the observed changes in energy transfer are via direct transnitrosation of MT. The reversal of L-SNCEE-mediated changes in FRET-MT by dithiothreitol provides further support for such nitrosative modifications to a cysteine residue(s) in MT, as DTT is reported to effectively remove thiol-bound NO groups from proteins [28; 286]. Alternatively, our methodology does not allow us to eliminate the possibility that a stereoselective mMino acid transport system, such as that recently described for SNC in PC12 cells [287], may also contribute to the differing effects of L- and D-SNCEE on FRET-MT.

We showed previously that FRET-MT was sensitive to bolus additions of buffer saturated with NO gas [60], whereas the present results show that •NO liberated from
D-SNCEE had no effect on this construct. The apparent discrepancy is most likely explained by exposure of the cells to much higher •NO concentrations in these earlier reports, which, under aerobic conditions, can form reactive species capable of nitrosating MT [288]. These conclusions are supported by the in vitro data demonstrating the S-nitrosation of purified MT protein in the presence of NO donors [124]. In summary, the collective evidence suggests that L-SNCEE alters the conformation of FRET-MT via transnitrosation of MT cysteine(s); however, our evidence for S-nitrosation remains indirect and it is therefore possible that an alternative DTT reversible mechanism could explain these effects.

Nitrosothiol-induced conformational changes in FRET-MT are consistent with previous reports both by our group [62; 124] and by others [56; 289] of NO-mediated release of metals from the thiolate clusters of metallothionein. The recent development of a FRET-based nanosensor based on the β-domain of recombinant metallothionein[289] may be useful in providing detailed structural information and quantification of zinc binding and release by the

reportedly more reactive [63; 290] of the two metal binding domains of MT in response to NO-related species. Unlike the genetically encoded GFPbased probes, however, introduction of this fluorescently labeled FRET indicator [289] into living cells will require microinjection techniques and as yet remains untested.

B.3.2 Activation of the cGMP reporter cygnet by SNCEE

It is believed that S-nitrosothiols activate sGC via decomposition and formation of •NO [291; 292; 293], which interacts with the iron–porphyrin center of the enzyme [294]. There has also been speculation regarding the direct activation of sGC by S-nitrosothiols [295; 296]. Our results, however, suggest that SNCEE activated PKGIa via an Fe–nitrosyl heme complex because cGMP production was sensitive to ODQ, which oxidizes the ferrous form of sGC to the ferric species and irreversibly inactivates the enzyme [297]. Furthermore, the scavenging of extracellular •NO by HbO_2 blocked the L-SNCEE-mediated effects on the cygnet-2 reporter. It should be noted that the concentration of SNCEE (50 μM) used in these experiments may generate •NO concentrations that are in excess of the reported EC50 for sGC, which ranges from 1 to 400 nM dependent upon the method of NO delivery [298]. If this is the case, then sGC could be maximally activated by only a small percentage of the total liberated •NO, and differences in the decomposition of L- versus D-SNCEE would not be detectable using our PKG-based FRET reporter. Pertinent to this discussion is the possibility that transnitrosation of MT occurs via breakdown of SNCEE and autoxidation of •NO. This chemistry will exhibit second-order dependence on the free [•NO], thus amplifying even modest differences in breakdown of L vs. D-SNCEE. However, our indirect determinations of •NO using the Greiss reaction to measure the stable decomposition products, NO_3^- and NO_2^-, indicate that L- and D-SNCEE generate

equivalent amounts of •NO under our experimental conditions.

B.3.3 FRET detection

FRET between GFP mutants offers a general mechanism to build genetically encoded indicators and to monitor dynamic molecular interactions in living systems. This report describes the use of spectral confocalbased imaging for FRET detection that is capable of providing full spectral information for each voxel of the fluorescent image. This obviates problems of signal bleedthrough and channel cross talk and permits detection of small, but potentially biologically meaningful, changes in FRET that are extremely difficult to resolve reliably using the more traditional methods relying on band-pass filters. We found that FRET detection using this spectral imaging approach is a highly effective technique for detecting posttranslation protein modifications induced by NO-related species in living cells.

B.4 ACKNOWLEDGEMENTS

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